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Cutaneous Epithelial Neoplasms of the Dog—A Report of 153 Cases

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A STUDY of canine skin tumors is not only of academic interest but also of practical value. It will benefit the practicing veterinarian who is confronted daily with the problems of diagnosing and treating cutaneous lesions of dogs. The objectives of this study were (1) to study the gross and microscopic features of the epithelial neoplasms of the canine skin; (2) to investigate their age, sex, breed, and site incidence; rate of growth; recurrence; and metastasis; and (3) to classify them on the basis of morphology, histogenesis, and biologic behavior.

MATERIALS AND METHODS

This study is based on the examination of cutaneous lesions from 7,489 dogs, collected by the Department of Veterinary Pathology during the ten-year period from January 1, 1949, to December 31, 1958. Approximately half of the accessions were from dogs necropsied in this department, and the other half consisted of formalized tissues submitted for diagnosis. All epithelial neoplasms of the skin were studied, excluding those of the modified glands (the mammary and perianal gland tumors). Tissues were fixed in 10 per cent neutral, buffered, isotonic formalin and stained routinely with hematoxylin and eosin. Special fixation of selected skin tissues was done in Helly's fluid; special stains or histochemical methods used were MacManus' periodic acid-Schiff, Masson trichrome, Mayer's mucicarmine, Wilder reticulum, Von Kossa's, Van Gieson's, Giemsa, and toluidine blue.

I. APOCRINE SWEAT GLAND TUMORS

Tumors originating in apocrine sweat glands occur in both man and dogs. They are of similar morphology and biologic behavior but are present in different parts of the body. In man, they have been described only in areas where apocrine sweat glands are normally present: the axillary, mammary papillary, umbilical, pubic, and anal regions, with the most frequently reported site being the vulva. In dogs, apocrine sweat glands occur throughout the entire hair-bearing skin.[10,31,37,44,51]

Review of Literature. Boucek,[5] in 1906, described an adenocarcinoma consisting of cuboidal cells lining the cystic spaces of a sweat gland in a dog. Cohrs,[6] in 1949, stated that sweat gland adenomas were rare in the canine skin and illustrated their papillary glandular pattern in a drawing.

Head,[18] in 1953, described 17 sweat gland tumors among 709 canine skin tumors found in a collection of 1,732 canine tumors made during a 12-year period. Cotchin,[7,8] in 1954, found 16 sweat gland tumors among 422 cutaneous canine tumors.

Innes,[21] in 1957, described an apocrine sweat gland adenoma in the foreleg of a 9-year-old Labrador Retriever. He also described a sweat gland carcinoma which had originated in the popliteal region and metastasized to the lungs in an 11-year-old Pug.

Lumen formation was present in all the canine sweat gland tumors reported by these authors. Other authors have considered certain basal cell tumors to be of sweat gland origin, a finding that cannot be supported by the observations in this study. The majority of human apocrine sweat gland tumors have a papillary pattern with lumina appearing as clefts or cysts. They are referred to as papillary hidradenomas and have recently been reviewed by Anderson,[4] Gates *et al.*,[16] Lennox,[29] Lund,[33] McDonald,[33] Novak and Stevenson,[39] and Teloh.[47]

Gross Lesions.—Pertinent data concerning 14 dogs with apocrine sweat gland tumors are summarized (table 1). The tumors were firm and sharply delineated, and

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TABLE 1—Fourteen Canine Apocrine Sweat Gland Tumors

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Characteristics
4996	.....	....	....	Elbow	1 x 1	Ulceration, papillary adenoma, tall cells.
5267	Cocker Spaniel	M	6	Hip	....	Papillary adenoma, thin stroma, ductal.
52609	.....	....	....	.....	....	Adenoma, two layers, ductal origin.
52843	.....	....	....	.....	....	Mixed adenoma, apices, cartilage.
54518	.....	....	....	Back	....	Adenocarcinoma, small cells, invasion.
54544	.....	....	....	Flank	....	Adenocarcinoma, apices, invasion.
541052	Terrier	....	9	Chest	10 x 8	Duration 1 yr., cystadenoma, spaces.
55755	Boxer	M	9	Chest	3 x 2	Adenocarcinoma, immature, mitoses.
56754	Bulldog	M	8	Hip	6 x 5	Papillary cystadenoma, apices, spaces.
56856	Boston Terrier	M	9	Head	2 x 1	Duration 1 yr., papillary adenocarcinoma.
561035	Cocker Spaniel	M	10	Stifle	3 x 2	Mixed adenocarcinoma, cartilage.
A157	Boston Terrier	F	7	Thigh	2 x 2	Mixed adenoma, apices, cartilage.
B625	Cocker Spaniel	M	6	Anus	1 x 1	Adenocarcinoma, invasion.
B1597	Cocker Spaniel	M	old	Chest	5 x 4	Adenocarcinoma, small lumina, invasion.

ranged in size from 1 by 1 cm. to 10 by 8 cm. Since tumors were found widely distributed in the skin of the head, trunk, and legs, this series of cases suggested no predominance in any particular cutaneous region. The dogs were 6 to 10 years old, with 4 Cocker Spaniels represented among the 9 dogs of known breed. Males predominated; 7 males and 1 female were listed in this series. The number of dogs, however, was too small to draw any definite conclusions as to any significant age, breed, and sex incidence.

Microscopic Appearance.—Three different morphologic diagnoses were used: adenoma, adenocarcinoma, and mixed tumors with cartilage.

The sweat gland adenoma was composed of glandular structures with lumina of variable sizes which had either one or two layers of lining cells. The lumina were large in the cyst adenomas, in contrast to the many narrow clefts consistently seen in the papillary adenomas. The tumors having a single layer of cells and showing occasional apices were believed to originate in the fundi of the sweat glands. The tumor cells were cylindrical, well-delineated, and had dense eosinophilic cytoplasms and large vesicular nuclei situated in the basal portion of the cells. Between the row of tumor cells and the dense collagenous basement membrane, a few fusiform nuclei of myo-epithelial cells were present. The tumors with two layers of tall cylindrical cells lining the lumina were considered to be of ductal origin (fig. 1).

The adenocarcinomas were often of the papillary type, with numerous projections of the anaplastic epithelium into lumina, which were filled with an eosinophilic fluid mixed with dead, desquamated, tumor epithelium. The tumor cells were large, cuboidal, and had dense eosinophilic cytoplasm which occasionally showed apices on the luminal side. The nuclei were large, vesicular, and spherical and had one or two swollen nucleoli. The supporting stroma was scant and was seen as narrow septa carrying the blood vessels between rows of tumor cells. There was no encapsulation visible microscopically, as tumorous glands were intermingled with normal collagen fibers of the dermis.

In the three mixed tumors (dogs 52843, 561035, and A157), the neoplastic epithelium formed a glandular pattern. Rows of single cells on thin connective tissue septa often formed papillary projections into the cyst-shaped lumina. Apices were often present on the luminal side of the tumor epithelium (fig. 2), while their basal surfaces blended with the underlying matrix. The tumors of dogs 52843 and A157 were diagnosed as mixed adenomas, and the more anaplastic tumor in dog 561035 was classified as a mixed adenocarcinoma. The cytoplasm was abundant, eosinophilic, and well delineated. The nuclei were large, and vesicular, and each usually had one large nucleolus. Mitotic figures were rare. The mesenchymal part of the tumor contained several areas of a bluish, reticulated, myxoid matrix which occasionally had changed into mature hyalin cartilage with ovoid, vesicular nuclei resting in small lacunae. The fibrous stroma occasionally had areas of hyalinized collagen, seen as a light pink, glassy, structureless mass surrounding an area of cartilage. The earliest visible metaplastic change in the stroma of the tumor appeared as a mucinous degeneration of collagen fibers (fig. 3). It occurred frequently in the immediate vicinity of anaplastic epithelium, but upon serial sectioning it was seen that the stroma some distance from tumor cells was changed. The normally pink eosinophilic collagen fibers

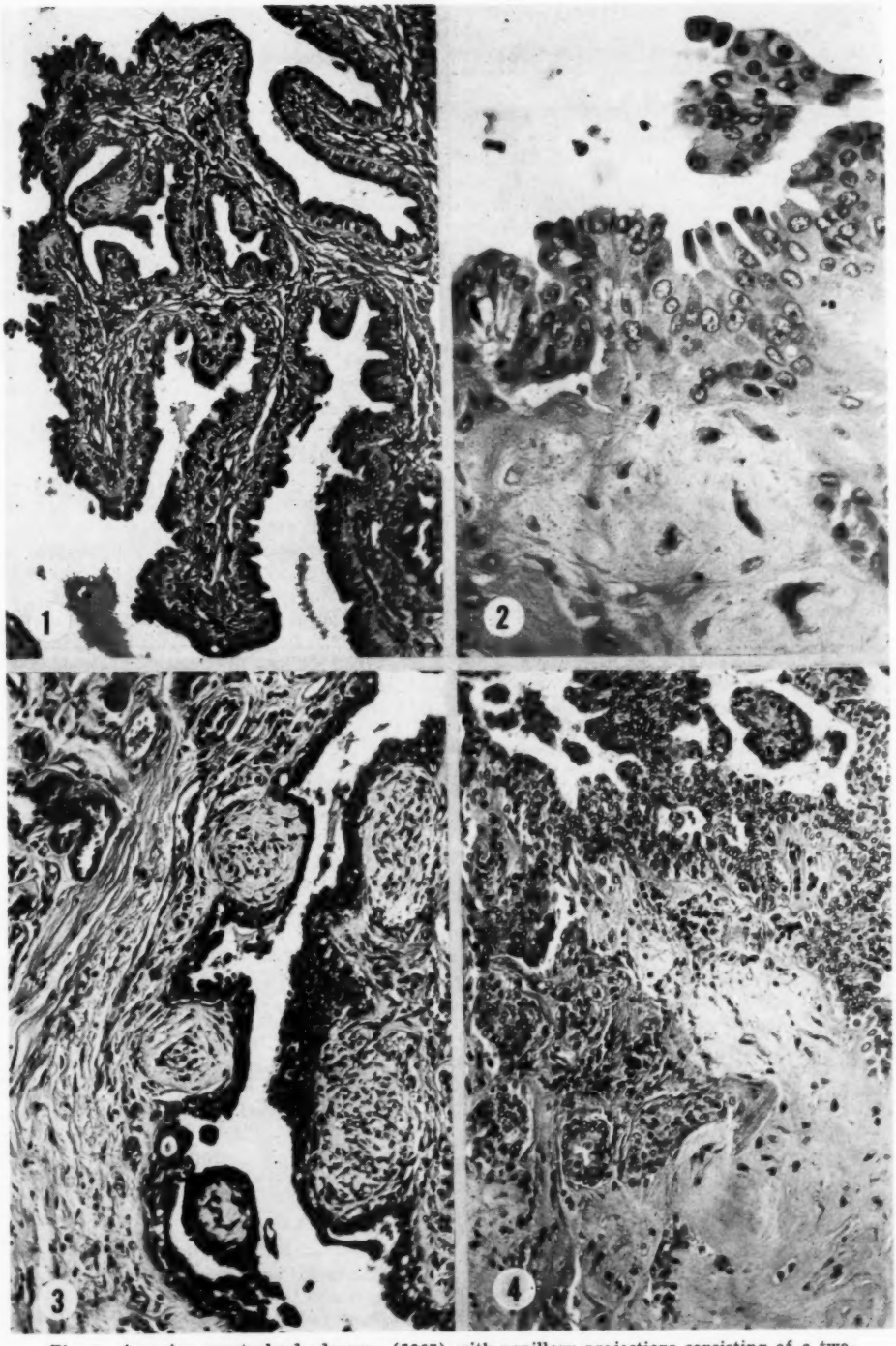

Fig. 1—Apocrine sweat gland adenoma (5267) with papillary projections consisting of a two-cell layer of tumor epithelium covering a fibrous stroma. H & E stain; x 248.

Fig. 2—Mixed apocrine sweat gland adenocarcinoma (561035) showing apex formation of the tumor epithelium and mucinous degeneration of the underlying stroma. H & E stain; x 173.

Fig. 3—Mixed apocrine sweat gland adenoma (A157) with several foci of mucinous degeneration of the stroma subjacent to the neoplastic duct epithelium. H & E stain; x 255.

Fig. 4—Mixed apocrine sweat gland adenocarcinoma (561035) with a large area of hyaline cartilage. H & E stain; x 255.

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became light blue, foamy, and reticulated, resembling mucinous degeneration. A few connective tissue cells (fibroblasts and histiocytes) and, possibly, myo-epithelial cells were imbedded in this newly formed mucinous matrix, which gradually changed into a homogeneous, hyalin matrix that was morphologically identical to cartilage (fig. 4). In addition, toluidine blue and Giemsa stains revealed a metachromatic reaction characteristic of hyalin cartilage.

Discussion.—The similarity of the disturbance of growth in apocrine sweat glands to hyperplasia and neoplasia in the mammary gland is to be expected, as both glands are modified cutaneous adnexa with apocrine development. The secretions of the mammary and apocrine sweat glands are similar morphologically, but obviously have a different histochemical composition. The similarity of sweat gland tumors to mammary tumors of the dog is a further indication of a close embryologic relationship between the two. This fact made it necessary to investigate the sites from which a few of the biopsy specimens were taken in order to rule out a possible mammary origin.

The apocrine sweat gland tumors of the dog closely resemble their counterpart in man. In both species, the tumors are usually grossly circumscribed, are located in the dermis, and grow in a papillary pattern.[1] Metastasis has been reported infrequently in man [45] and only once in a dog.[21]

In this study, chondroid metaplasia was found in three mixed apocrine sweat gland tumors. The chondroid tissue had the morphologic and tinctorial characteristics of mature hyalin cartilage, as demonstrated by metachromatic staining. Mixed tumors occur with great frequency in the mammary glands of dogs. The literature contains one reference to a mixed thyroid tumor,[52] while no reports of mixed tumors in other sites of the dog were found. In the skin of man, mixed tumors are present in the salivary, lacrimal, mammary, and sweat glands. The occurrence of cartilage in a predominantly epithelial tumor is attributed to metaplasia, presumably due to a changed metabolism of the tumor tissue. The exact process is unknown, and three different theories have been postulated. Allen [2,3] claims that the nuclei in the cartilage matrix are detached epithelial cells that have become chondrocytes by a dysembryogenetic change. A second theory, supported by Erichsen [12] and Cotchin,[9] is that the mesenchymal matrix is produced by the myo-epithelial cells surrounding the tumorous epithelium. A third theory, in which the metaplasia is considered to be a pure mesenchymal change, has been supported by Mulligan [35] and Moulton.[34]. In available material from mixed tumors of thyroid, sweat, mammary, and perianal glands of dogs, the earliest stages of metaplasia suggest that the nuclei present in the cartilage lacunae are of mesenchymal or myo-epithelial origin. The metaplasia appears to be a degenerative process of the connective tissue stroma, caused by an altered metabolism due to the presence of rapidly growing tumor cells.

Summary and Conclusions.—Fourteen tumors of apocrine sweat glands were described. Six were classified as adenocarcinomas, five as adenomas, and three as mixed tumors. The three mixed tumors appeared to be the first such neoplasms of apocrine gland origin to be described in the dog. No age, breed, or site preference was recorded. The similarity between human and canine apocrine tumors was recognized.

II. BASAL CELL TUMORS

This neoplasm of the skin has been called basal cell carcinoma, basal cell epithelioma, and basalioma. It originates in the basal cell layer of the epidermis, in hair follicles, or in embryonal cells in the dermis. Despite its origin in undifferentiated cells with great growth potential, it is classified as a benign neoplasm; in man and dogs, however, metastasis has been described in rare instances. In this study, the term "basal cell tumor" has been used to designate epithelial tumors of the canine skin that did not provide evidence of differentiation. If differentiation occurred in the direction of normal structures (hair follicles, sebaceous glands, or sweat glands), the tumor was not included under the heading of basal cell tumor but under its corresponding category.

Review of Literature.—Canine basal cell tumors have been described by Feldman,[13] who, in 1932, mentioned two, one on the face and one on the shoulder. Microscopically, both tumors consisted of long, slender, irregular, tortuous columns of basal cells surrounded by dense fibrous tissue.

Jackson,[22] in 1936, described 8 dogs with basal cell tumors, four of which occurred

TABLE 2—Thirty-Three Canine Cutaneous Basal Cell Tumors

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Characteristics
4952	Mixed breeding	SF*	7	Cheek	....	Serpentine strands.
51432	.....	....	....	Ear	1.0 x 1.0	Solid masses, serpentine strands.
531296	Hound	M	9	Chest	9.0 x 5.0	Serpentine strands.
54325	.....	....	....	Cheek	....	Solid masses, serpentine strands.
54367	.....	....	....	Submaxilla	....	Serpentine strands.
54450	.....	....	....	Elbow	....	Serpentine strands.
54624	Brittany Spaniel	M	4	Chest	5.0 x 4.0	Strands, solid fusiform cells.
54944	Airedale Terrier	M	8	Temporal	1.0 x 1.0	Duration 2 mo., strands, scirrhous.
541111	Chow Chow	M	9	Chest	....	Solid, serpentine strands.
55186	Springer Spaniel	SF	9	Elbow	....	Serpentine strands, scirrhous.
551146	Cocker Spaniel	F	6	Ear	2.0 x 1.0	Duration 2 mo., strands.
551254	Collie	F	6	Maxilla	1.0 x 1.0	Recurrence, solid masses.
551255	.....	F	7	Submaxilla	3.0 x 3.0	Solid masses, mitoses.
551268	Shepherd	....	4	Axilla	6.0 x 4.0	Solid islands, mitoses.
551971	Chow Chow	M	14	Temporal	5.0 x 2.0	Duration 2 mo., cellular strands.
56569	Spaniel	M	3	Occiput	1.0 x 1.0	Duration 2 mo., serpentine strands.
56812	Mixed breeding	F	8	Cheek	2.0 x 1.0	Recurrence, scirrhous, medusoid heads.
561484	Mixed breeding	F	8	Mandible	2.0 x 2.0	Solid cords, mitoses.
561499	Cocker Spaniel	F	12	Temporal	3.0 x 2.0	Recurrence 7 mo., solid masses.
A753	Collie	M	8	Neck	5.0 x 5.0	Serpentine strands, solid masses.
A901	Cocker Spaniel	M	6	Neck	4.0 x 2.0	Serpentine strands.
A1474	.....	F	11	Cheek	1.0 x 1.0	Serpentine strands, solid masses.
A1446	English Setter	....	....	Neck	....	Serpentine strands, medusoid heads.
A1731	Airedale Terrier	F	6	.....	4.0 x 4.0	Slow growth, serpentine strands.
B117	Cocker Spaniel	M	8	Ear	3.0 x 2.0	Duration 3 mo., serpentine strands.
B321	Mixed breeding	M	4	Temporal	6.0 x 6.0	Duration 3 mo., serpentine strands.
B894	Boxer	M	8	Eyelid	0.5 x 0.5	Solid masses, melanin.
B1001	.....	M	5	Shoulder	1.0 x 0.5	Duration 3 mo., solid masss.
B1320	Cocker Spaniel	F	7	Ear	3.0 x 2.0	Serpentine strands, solid masses.
B1379	German Shepherd Dog	M	8	Neck	5.0 x 4.0	Duration 1 mo., serpentine strands.
B1540	Collie	F	7	Submaxilla	4.0 x 2.0	Serpentine strands, medusoid.
B1663	Cocker Spaniel	F	3	Neck	1.0 x 1.0	Solid masses, inclusion cyst.
B1899	.....	....	....	Ear	2.0 x 2.0	Serpentine strands.

* SF = spayed female.

on the face. Two different histologic patterns prevailed, one having strands and the other having solid masses of basal cells. Willis,[52] in 1938, stated that sweat and apocrine glands are the principal source of basal cell and adenoid skin tumors of the dog. Two photomicrographs of a basal cell tumor from the neck of a mongrel illustrated the ribbon type.[52]

Head,[18] in 1953, found 49 basal cell tumors among 709 cutaneous and subcutaneous tumors in a collection of 1,732 canine tumors diagnosed in a 12-year period. The 49 tumors were classified as superficial or subepidermal. Of the 28 superficial tumors, three recurred and three metastasized. The 21 subepidermal tumors had medusoid structures and were located predominantly on the head and neck. Three of these recurred but none metastasized. Mulligan,[35] in 1949, grouped the basal cell tumors with hair follicle tumors and sweat gland tumors. Two of his photomicrographs (plate 21, B and C) of tumors diagnosed as sweat gland carcinomas illustrated a pattern which, in this study, identifies the ribbon type of basal cell tumors.

Cohrs,[6] in 1954, mentioned the frequency of basal cell tumors in the neck and back region of the dog and illustrated their morphology in a drawing of the ribbon type of this tumor. Groth,[17] in 1955, described four basal cell tumors in male dogs as basaliomas. They were seen during pathologic examinations of tissues from 1,566 dogs. Three of them occurred on the head and, microscopically, the ribbon type was evident.

Smith and Jones,[43] in 1957, mentioned the occurrence of a solid type and a type composed of rows of cells, designated as apocrine sweat gland carcinoma.

Gross Lesions.—Thirty-three basal cell tumors were available for study (table 2). The tumors were firm, round masses, varying in size from 0.5 by 0.5 cm. to 9.0 by 9.0 cm. They appeared to be encapsulated and well demarcated from the underlying tissues (fig. 5) and were firmly attached to the skin, which occasionally was ulcerated. The cut surfaces were uniformly white and firm. The distribution of the tumors in the skin was of particular interest; of 32 (80%), for which the sites were given, 26 were located in the head and neck area. The site of one tumor was not indicated. There were 13 males and 13 females. The ages ranged from 3 to 14 years with the average age being 7 years. The Spaniel-type dog was the breed most frequently represented;

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9 of 25 dogs in which the breed was known or 36 per cent of the dogs with basal cell tumors were Spaniels. Nineteen per cent of the canine population in this geographical area during 1956–1957 were Cocker Spaniels.[38] Three tumors recurred and they all contained predominantly solid areas of basal cells. The recurrence was attributed to incomplete surgical excision of the invading masses of tumor cells in the corium. Neither vascular invasion nor metastases were found in the 33 tumors described in this study.

Microscopic Appearance.—The basal cell tumor was composed of small basaloid cells with large ovoid nuclei, often arranged in a palisading manner with the long axis of the nuclei placed perpendicular to the basement membrane formed by the surrounding collagenous tissue. The nuclei were rich in fine, dust-like chromatin and had no visible nucleoli. The cytoplasm stained faintly with eosin, probably due to a low protein content. It appeared reticulated, and no cytoplasmic outline was visible. Two main histologic patterns were seen, the ribbon and the solid forms. However, all stages of transition were present between these two, even within the same tumor. The ribbon type had long, narrow, serpentine strands of cells winding themselves through the fibrous connective tissue of the dermis and subcutis (fig. 6). These strands consisted of one, two, or three rows of palisading nuclei. Junctions of strands were frequently seen, with the formation of isolated islands of stroma surrounded by two rows of cylindrical cells. The stroma that had been trapped in this manner was often altered by mucinous degeneration or liquefaction. When such changes occurred, a resemblance to a glandular tumor was apparent. The term "adenoid basal cell tumor" has been used to describe this lesion in human dermatopathology.[29] Another stromal change was observed in some tumors of the ribbon type; collagenous connective tissue surrounding the epithelial strand became very abundant and dense, with a glassy appearance resembling hyalin. The solid form (fig. 7) had predominantly large, round, or oblong masses of small basaloid cells which, at the perimeter, were occasionally lined up in a palisading manner. The connective tissue next to the peripheral layer occasionally appeared as a basement membrane. In more actively growing areas, the cells were fusiform and their nuclei contained mitoses. The connective tissue surrounding such masses was not differentiated into a basement membrane and appeared to be invaded by the basaloid cells. Both the ribbon and the solid forms of the tumor projected into the subcutis and invaded the surrounding dermis, necessitating wide surgical excision. Occasionally, solid areas of basaloid cells were mixed with epithelial strands which were seen to project from the solid islands of cells like the tentacles of an octopus. Such cell formations were not unusual, and they have been referred to as Medusa heads (fig. 8).

Discussion.—The findings presented in this study are in agreement with all but two [35, 43] of the reports found in the literature on canine cutaneous tumors. In those two reports, the term "apocrine sweat gland tumor" was used. The resemblance of an adenoid basal cell tumor to a sweat gland tumor is only superficial, as the spaces surrounded by basal cells in the ribbon type represent engulfment of stroma and not a true glandular lumen. The term "basal cell tumor" was chosen for this group of cutaneous neoplasms because no differentiation toward any of the epidermal adnexa could be demonstrated, even when multiple sections were prepared. The tumor was considered to originate in the basal cells of the stratum germinativum epidermis, in the hair follicle, or from latent embryonal epithelial cells. This latter theory has gained much popularity in recent years and is supported by Lever,[29] Pinkus,[41] and Swerdlow.[46] The high incidence of canine basal cell tumors occurring on the head and neck agreed with the site incidence of the basal cell tumor in man. According to Allen,[2] 87 per cent of human basal cell tumors occur in the skin of the head and neck. The ribbon type, which is the most frequent form in the dog, is also present in man, as described by Foot [15] and Lund,[32] where it represents the least aggressive form. The findings in this study and those reported by Head [18] indicate that the same is true for the dog. The solid form of basal cell tumor in the dog is capable of recurring and metastasizing.

Conclusion and Summary.—Thirty-three basal cell tumors occurred predominantly in old dogs with an average age of 7 years. They affected males and females with equal frequency, and no particular breed preference was evident. The skin of head and

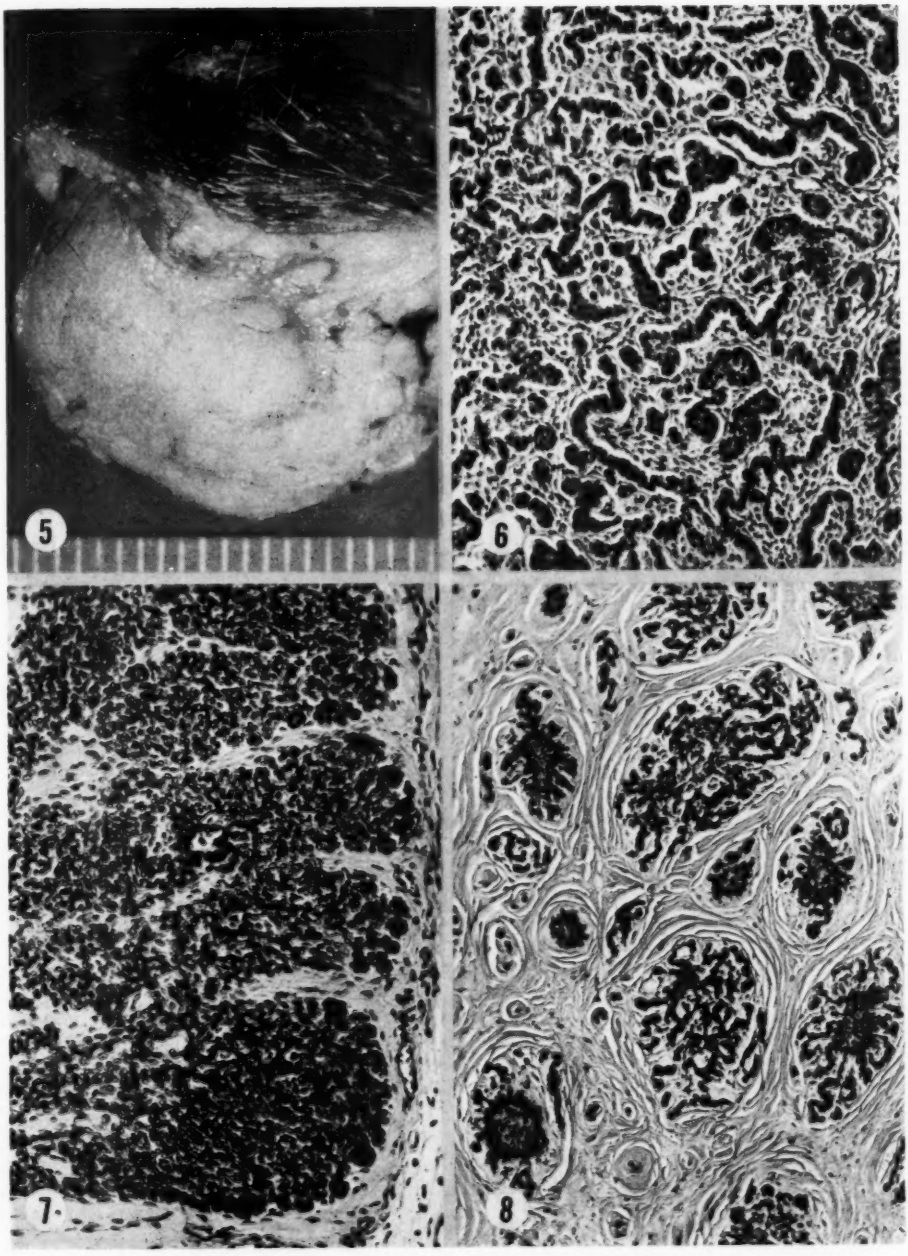

Fig. 5—Basal cell tumor (561484) illustrating the characteristic spherical shape, encapsulation, and attachment to the skin. Millimeter units are indicated at the bottom.

Fig. 6—Basal cell tumor (551971) depicting the ribbon pattern. Serpentine strands of palisading, ovoid hyperchromatic nuclei are surrounded by a cellular corium. H & E stain; x 240.

Fig. 7—Basal cell tumor (54624) depicting the solid pattern, with cellular masses of nonpolar nuclei containing many mitoses. H & E stain; x 255.

Fig. 8—Basal cell tumor (56812) with cells arranged in medusoid pattern among the hyalinized collagen fibers of the corium. H & E stain; x 182.

neck showed a pronounced predisposition, with 80 per cent of the tumors occurring in this area. The tumors were round, firm, and movable over the underlying tissue but were firmly connected with the skin. Despite a tendency to local invasion, metastases were absent in this material, and the tumor recurred in only 3 dogs, probably as the result of incomplete excision. Two different patterns of the basal cell tumor were recognized, the ribbon and the solid types, with the latter being more aggressive and more apt to recur. The ribbon type has been classified by some authors as a sweat gland tumor because of certain morphologic similarities.

III. Hair Matrix Tumors

Trichoepithelioma

Epithelial tumors, forming a pattern simulating either mature or incompletely formed hair follicles, are named trichoepithelioma, hairmatrixoma, or trichomatrioma.[20]

Review of Literature.—The trichoepithelioma of dogs has not attracted much attention in the literature. Mulligan[35] classifies the hair follicle tumor under the heading of basal cell carcinoma. Smith and Jones[43] mention the presence of trichoepithelioma in the skin of dogs and indicate its resemblance to the human counterpart. They also consider this tumor to be related to the calcifying epithelioma.

Gross Appearance.—Seven trichoepitheliomas were found throughout the skin of the body without any particular site preference (table 3). They appeared as very firm, well-circumscribed, round or ovoid masses, easily movable over the underlying muscle, tendon, or bone. The epidermis covering the tumors was atrophic, hairless, and often ulcerated, probably due to mechanical traumatization, as no tumorous invasion of the epidermis was seen. The cross section appeared gray with occasional white areas, indicating large keratinized cell masses (fig. 9). The size ranged from 1 by 1 cm. to 8 by 6 cm. The dogs averaged 10 years of age and there was a slight majority of females. Recurrences did not follow excision, and no metastases were found in the 7 dogs described in this study.

Microscopic Appearance.—Varying degrees of differentiation were demonstrated, from a predominantly undifferentiated basaloid tumor to the well-differentiated form with numerous, rather well-formed hair follicles. Case A1038 was an example of the poorly differentiated type which only differed from the basal cell tumor by having several foci of central keratinization in its narrow, solid cords of small basaloid cells (fig. 10). The ovoid, large nuclei were seen to change their orientation from perpendicular to the long axis of the cord to a concentric arrangement around foci of keratinization. The connective tissue stroma surrounding the cords was loose and fibrillar, and occasionally mucinous degeneration was indicated by a foamy, bluish appearance.

The majority of trichoepitheliomas were in intermediate stages of differentiation with large, solid masses of small basaloid cells having a small amount of faintly eosinophilic, poorly demarcated cytoplasm. Their nuclei were large and ovoid and contained dust-like chromatin. These solid masses had scattered central areas of keratinization with concentrically arranged flakes of cornified epithelium (fig. 11). The transition between the basaloid cells and the centrally located keratinized cells was always extremely abrupt, and, in a few of the larger keratinized areas, ghost cells were present (fig. 12). Occasional blue granules

TABLE 3—Seven Canine Cutaneous Trichoepitheliomas

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Characteristics
541771	Dalmatian	F	10	Cheek	2 x 2	Duration 2 mo., solid masses of small basaloid cells, few follicles.
55420	Dalmatian	F	11	Shoulder	8 x 8	Duration 1 mo., numerous follicles, heavy keratinization, early mineralization.
56394	Springer Spaniel	F	12	Head	2 x 2	Lobules of heavily keratinized masses, melanin, early mineralization.
56919	Dachshund	F	8	Foreleg	2 x 2	Solid areas of small basaloid cells, few follicles.
A1038	Brittany Spaniel	SF*	10	Chest	3 x 2	Strands of solid cells, occasional keratinization.
B194	Cocker Spaniel	M	9	Ear	3 x 2	Encapsulated, lobules of solid basaloid cells, occasional keratinization.
B753	English Shepherd	M	8	Tail	6 x 5	Duration 1 yr., ulceration, solid masses, central mummification.

* SF = spayed female.

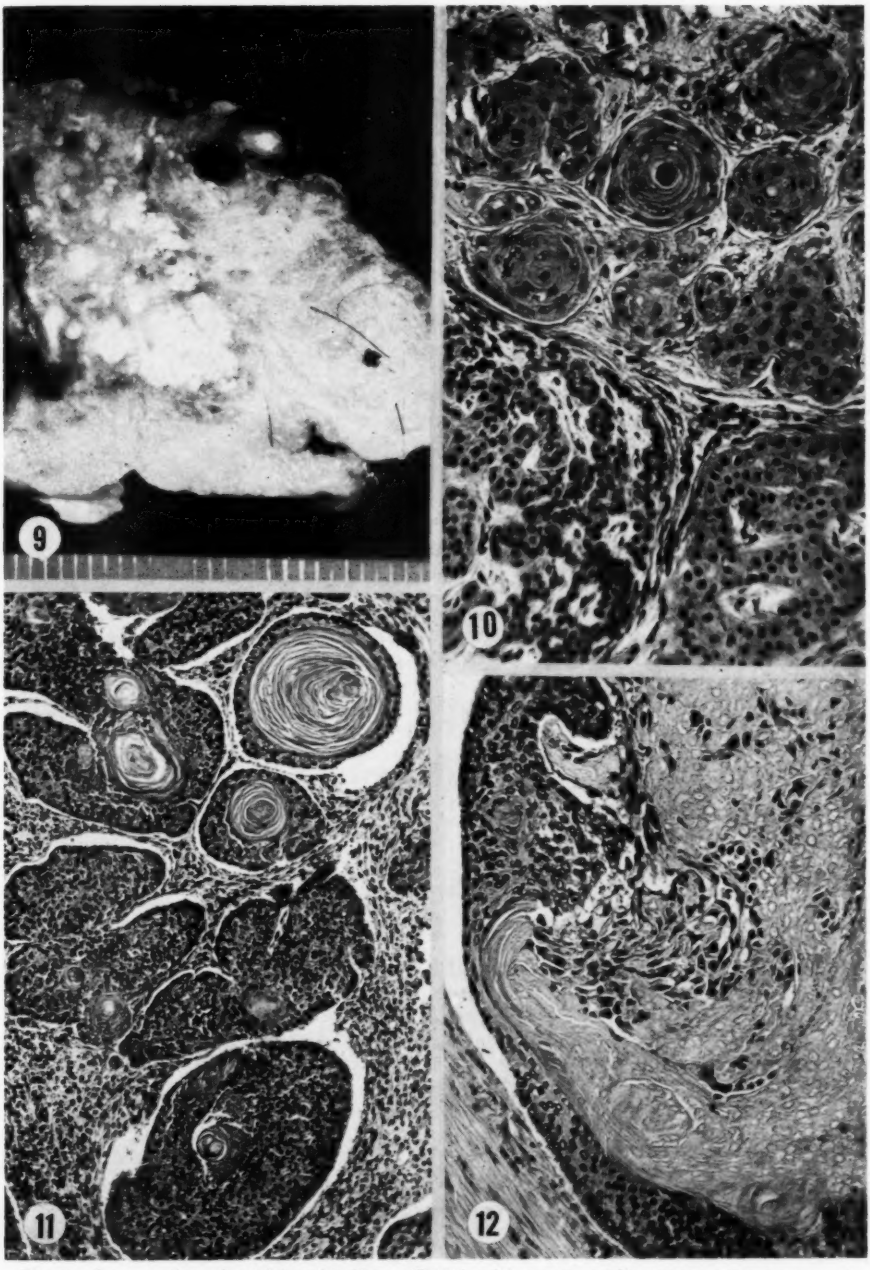

Fig. 9—Cross section of a trichoepithelioma (B753) with creamy white areas of keratinization. Millimeter units are indicated at the bottom.

Fig. 10—Trichoepithelioma (A1038) with strands of undifferentiated basaloid cells below and a few areas of keratinization above, indicating hair follicle differentiation. H & E stain; x 360.

Fig. 11—Trichoepithelioma (B753) with some keratinization present centrally in the islands of basaloid cells. The surrounding corum is infiltrated with lymphocytes and plasma cells. H & E stain; x 275.

Fig. 12—Trichoepithelioma (B753) with mummification; from the same tumor as shown in figure 11. Notice the ghost cells and the abrupt line of cornification. H & E stain; x 240.

were present in the keratinized pink masses, indicating early mineralization.

Discussion.—The trichoepitheliomas were differentiated from squamous cell carcinomas by the smaller size of the cells; their scant, indistinctly outlined, lightly stained cytoplsm; the lack of intercellular bridges; the lack of invasion; and the abruptness of the keratinization. A squamous cell or epidermoid carcinoma has larger cells, and the keratinization and pearl formation is always a gradual process with several layers of tumor cells undergoing transitional changes. As transitional forms between a trichoepithelioma and a basal cell tumor exists, the differential diagnosis depends on the presence of focal areas of keratinization. The prognosis for a trichoepithelioma is excellent, but a basal cell tumor may recur if the surgical excision is not made sufficiently wide.

CALCIFYING EPITHELIOMA

The calcifying epithelioma of canine and human skin has many features in common with the trichoepithelioma. It has been considered to be of hair matrix origin, probably representing a late stage in which cellular activity ceased and mineralization has occurred. This tumor was first described in man by Malherbe, in 1880, and has since been known as epithelioma of Malherbe, self-limiting epithelioma, and mummifying epithelioma.

Review of Literature.—As its histiogenic origin is obscure, various possibilities have been postulated. Eller and Eller [11] attributed its formation to an embryonal developmental disturbance; Allen [2] believed it originated in a sebaceous cyst. Warvi and Gates [50] and King [23] mentioned that it was probably a late stage of an epidermal inclusion cyst. Tilden [48] and Kuang-Yu [25] considered the possibility that the calcifying epithelioma may be a basal cell tumor. Others believe the calcifying epithelioma to be the end stage of a trichoepithelioma.[19,29,49]

In dogs, only three reports of calcifying epitheliomas were found. Head,[18] in 1953, found 14 lesions, designated collectively as calcinoses or calcified epitheliomas, among 709 canine skin tumors.

Smith and Jones,[43] in 1957, published illustrations of two calcifying epitheliomas, one occurring in a Kerry Blue Terrier and the other in a Poodle. They mentioned the difference between this tumor and the calcified dermoid cyst.

Sandersleben,[42] in 1958, described two cases, one on the back of an 8-year-old male Schnauzer and the other on the chest of a 5-year-old Fox Terrier. The author stated that both tumors differed from so-called calcinosis circumscripta.

Gross Lesions.—The five calcifying epitheliomas encountered did not vary significantly from the gross appearance of the trichoepithelioma. They ranged in size from 2 by 2 cm. to 6 by 5 cm. and were located on the legs and shoulder (table 4). The overlying epidermis was atrophic and often ulcerated, probably due to mechanical trauma from rubbing. The cut surface was lobulated and was characterized by white, chalky areas of mineralization.

Microscopic Appearance.—A characteristic of this tumor was the presence of large areas of a peculiar coagulation necrosis in the masses of neoplastic epithelial cells. This process has been called mummification by some authors and is unique in hair matrix tumors. The viable cells from which this tumor originated were of the same size, morphology, and staining ability as those of the trichoepithelioma. It was the degree of mummification and subsequent mineralization that separated this tumor from the trichoepithelioma. The calcifying epithelioma generally attained a large size before central necrosis occurred but, despite this active cell proliferation, no invasive tend-

TABLE 4—Five Canine Calcifying Epitheliomas

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Characteristics
51512	Kerry Blue Terrier	....	2	Shoulder	3 x 2	Several foci of basophilic basal cells concentrically arranged around necrotic calcified centers.
5294	Kerry Blue Terrier	M	4	Metatarsus	2 x 2	Large solid masses of basophilic cells, necrosis and calcification.
54924	Kerry Blue Terrier	F	8	Radius	2 x 2	Multilobulated cysts with keratinized necrotic debris undergoing calcification, ghost cells.
541294	.....	....	....	.....	....	Several keratinized cysts with central mineralization; basaloid cells at periphery.
B1224	Cocker Spaniel	M	9	Elbow	6 x 5	Central keratinization, keratolysis, early mineralization, encapsulated, ulceration.

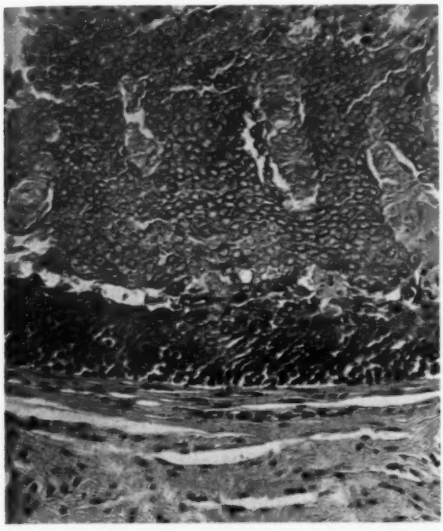

Fig. 13—Calcifying epithelioma (51512) with a rim of small, dark, basaloid cells surrounding a mummified central area containing numerous ghost cells. H & E stain; x 360.

Fig. 14—A nodule of calcinosis circumscripta showing the characteristic cross section above and a circular ulcer below. Notice the similarity to figure 9. Millimeter units are indicated at the bottom.

ency was seen. In some of the cell masses where advanced mummification and mineralization were present, only a narrow, peripheral rim of basal cells could be detected (fig. 13). By searching through the pink eosinophilic centers, several areas of so-called ghost cells were recognized, with their nuclear outline still preserved. One tumor (54924) had areas which appeared to be osteoid tissue with individual nuclei situated in lacunae within a dense eosinophilic matrix. In the completely keratinized and mineralized lobules of the tumor, where no viable and stainable cells were visible, a foreign body reaction was present in the surrounding connective tissue. Large, multinucleated, giant cells were seen in apposition to mineralized keratin debris.

Discussion.—The differentiation between a completely calcified epithelioma and a calcified epidermal or dermal inclusion cyst is difficult. The presence of ghost cells and a peripheral rim of a multiple cell layer of small basal cells rule out mineralized cysts and establish a diagnosis of calcifying epithelioma.

The similarity between a completely mineralized calcifying epithelioma (54924) and other forms of nodular mineralization in the skin of dogs is interesting. Such nodules have been referred to as calcinosis circumscripta by Smith and Jones,[43] while the German authors Cohrs,[6] Kretzschmar,[24] Kunze,[26] and Pallaske [40] use the term *Kalkgicht*, which, translated, is calcium gout (fig. 14). The lesions consist of sharply demarcated lobules of mineral deposits surrounded by a foreign body reaction with multinucleated giant cells and fibrosis. As the peripheral rim of basal cells in an old calcifying epithelioma may have necrotized and mineralized, only the presence of ghost cells somewhere in the mineralized mass will differentiate it from other types of cutaneous nodular mineralization. It is, therefore, important to make a careful histologic study of all calcified nodules in the skin of dogs in order to rule out old calcifying epitheliomas. This point may be of greater importance in dogs than in man, since a skin nodule is apt to remain untreated for a longer period in the former and thus have time to undergo total mineralization.

In this study, 3 Kerry Blue Terriers were among the dogs with calcifying epitheliomas. The illustrations presented by Smith and Jones [43] in figure 59 were from

a Kerry Blue Terrier and the prescapular region of a Poodle. Although the known number of dogs with calcifying epitheliomas is small, their occurrence in Kerry Blue Terriers may eventually prove to be more than coincidental. The normal anatomy of the skin of Kerry Blue Terriers and Poodles is similar and differs from other breeds of dogs. Their epidermis is thin and each follicle contains a greater number of hairs.

Summary and Conclusions.—The canine trichoepitheliomas studied were benign neoplasms which mimicked hair follicle architecture and were considered to have originated in the hair matrix. The transition forms common to these tumors and to calcifying epitheliomas were indicative of a common origin, but with a different tendency to mummification. Both were firm, spherical, and well demarcated, without a tendency to metastasize or recur. Old dogs of an average of seven years were most frequently affected. The Kerry Blue Terrier was the breed that most frequently developed the calcifying epithelioma. All tumors, where the site is recorded, were located in the dermis of the legs and shoulder.

IV. EPIDERMOID TUMORS

SQUAMOUS CELL CARCINOMA

The squamous cell or epidermoid carcinoma has great tendencies to ulceration, invasion, recurrence after surgical removal, and lymphatic metastasis. In human skin, this tumor has a preference for certain sites; *i.e.*, the exposed parts of the face, ears, and the dorsum of the hands. A number of chemical carcinogens such as pitch, tar, soot, and arsenicals are known to produce epidermal lesions that eventually may result in squamous cell carcinomas. Physical causes are exposure to x-rays, ultraviolet light, and radioactive substances. Chronic irritation, resulting in nonhealing fistulas and ulcers, is also known to predispose animals to squamous cell carcinomas.

Review of Literature.—Folger,[14] in 1917, reviewed the world's literature on tumors of animals and found descriptions of 13 squamous cell carcinomas of the skin of dogs.

Murray [36] describes three squamous cell carcinomas among 21 cutaneous tumors of dogs, one occurring on the foreleg and one in the anal region.

Feldman,[13] in 1932, found nine cutaneous carcinomas among 78 canine tumors and quoted the findings of Lubarsch, who found 143 cutaneous carcinomas among 784 canine carcinomas; of Sticker, who found 166 cutaneous carcinomas among 766 canine carcinomas; and of Schütz, who found 14 cutaneous carcinomas during 1,241 canine necropsies in a 14-year period. Unfortunately, the histologic type was not specified in any of these reports.

Jackson,[22] in 1936, described squamous cell carcinomas under the term "acanthoma" and stated that they occurred throughout the skin, with the exception of

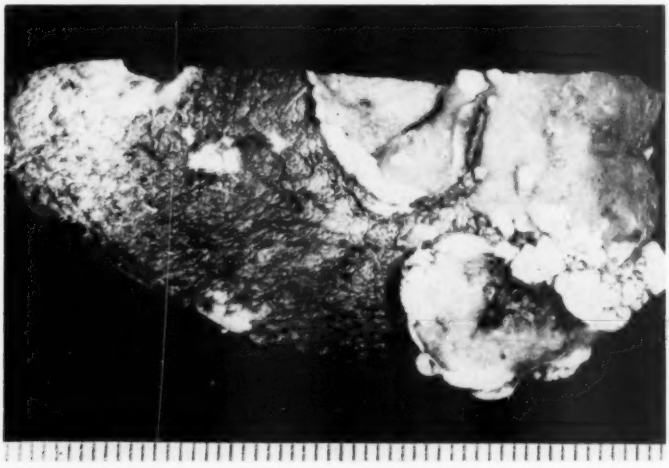

Fig. 15—A squamous cell carcinoma (561047) showing several fungoid protruding ulcers of the epidermis.

that of the head, and that no metastases were seen in his material.

Rudduck and Willis,[52] in 1938, described a squamous cell carcinoma of the elbow with metastases to the axillary lymph nodes in a 9-year-old male German Shepherd Dog.

Mulligan,[35] in 1949, found 17 squamous cell carcinomas and seven papillomas among 283 cutaneous epithelial neoplasms. They were found in 16 males and 7 females. Eighteen tumors occurred in animals 7 to 12 years of age. Head,[18] in 1953, described 36 squamous cell carcinomas among 709 cutaneous canine tumors. Cotchin,[7,8] in 1954, described 48 squamous cell carcinomas among 932 cutaneous canine tumors. Larsson,[27] in 1956, found 19 squamous cell carcinomas among 266 cutaneous tumors encountered in a two-year period.

Gross Lesions.—The 31 dogs with squamous cell carcinoma in this study (table 5) were 5 to 16 years old. There was no indication of breed predilection among the 18 females and 13 males. The tumors were hard, irregular in outline, and not encapsulated; they frequently protruded from the body surface as a cluster of ulcerated, fungoid nodules (fig. 15). Recurrence after surgical removal took place in 3 dogs. Seventeen tumors were seen, either microscopically or grossly, to invade lymphatics or deeper tissues. In only one instance was a metastasis found clinically in the corresponding regional lymph node. This, however, was probably not a true indication of the actual number of metastases, as follow-up studies were not obtained for many of the earlier cases. The sites of the squamous cell carcinomas were scattered throughout the skin of the entire body (table 5). Seven tumors were present on the feet, while only three were located on the head and neck.

Microscopic Appearance.—The presence of a squamous cell carcinoma was invariably associated with much inflammatory reaction of the connective tissue stroma and of the surrounding dermis. The more mature carcinomas, with much pearl formation, were often accompanied by many lymphocytes and plasma cells, while the anaplastic type was seen to infiltrate the collagen fibers without inciting such inflammatory reaction (fig. 16).

In 1 dog, a continuation could be demonstrated from the hyperplastic rete ridges of the epidermis to the strands of squamous cell carcinoma invading the dermis. The epidermis in the immediate vicinity of the neoplasm was found to have an epitheliomatous hyperplasia with greatly thickened

TABLE 5—Thirty-One Canine Cutaneous Squamous Cell Carcinomas

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Grade and characteristics
49101	Fox Hound	M	11	Shoulder	2 x 1	I, pearls.
5141	Boston Terrier	F	10	Hind toe	1 x 1	II, duration 7 mo.
51212	Beagle	F	12	Neck	6 x 4	III, invasion.
5251	Mixed breeding	F	12	Gluteal	4 x 3	I, duration 3 mo., pearls.
52751	Pointer	....	8	Sacral	....	III, invasion.
52938	Scottish Terrier	F	13	Toe	2 x 1	III, invasion.
521032	Mixed breeding	M	7	Hind foot	....	II, small cells.
54487	Cocker Spaniel	M	8	Lumbar	....	II, duration 2 mo., recurred.
54498	.....	M	14	Prepuce	....	II, recurrence 2 mo.
5530	Irish Setter	F	14	Sacral	....	III, invasion, mitoses.
55934	German Shepherd Dog	F	13	Hindleg	3 x 3	II, duration 6 mo., invasion.
55960	Scottish Terrier	F	12	Toe	2 x 2	III, duration 6 mo., bone invasion.
551922	Norwegian Elkhound	F	10	Thigh	1 x 1	I, pearls, lymphocytes.
551950	Scottish Terrier	....	....	Scapula	1 x 1	II, invasion.
56802	Irish Setter	F	10	Back	4 x 2	I, pearls, inflammation.
561047	English Setter	M	8	Inguinal	5 x 3	II, slight invasion.
561066	Springer Spaniel	SF*	13	Tarsus	2 x 1	II, invasion, plasma cells.
561559	Collie	M	6	Shoulder	5 x 5	I, duration 6 mo., pearls.
A317	Scottish Terrier	M	8	Abdomen	4 x 2	III, invasion.
A617	Collie	F	12	Axilla	10 x 2	IV, anaplasia, lymphatic invasion.
A298	Cocker Spaniel	F	7	Foot pad	3 x 2	II, invasion.
A1049	Cocker Spaniel	SF	8	Metatarsus	2 x 2	III, recurrence, invasion.
A1151	Boxer	M	5	Scrotum	3 x 2	II, invasion.
A1280	Pomeranian	M	6	Tail	1 x 1	II, rapid growth.
A1468	Collie	M	10	Foot pad	4 x 1	II, invasion, lymphocytes.
B135	German Shepherd Dog	F	8	Cheek	4 x 4	II, necrosis.
B288	Cocker Spaniel	F	8	Hip	3 x 2	II, duration 7 mo., invasion, ulceration.
B806	.....	F	16	Abdomen	6 x 5	III, invasion, anaplasia.
B1088	Poodle	M	10	Toe	2 x 1	I, pearls
B1129	German Shepherd Dog	M	15	Flank	5 x 5	II, ulceration, invasion.
B1323	Terrier	M	12	Jaw	4 x 4	III, duration 6 mo., metastasis.

* SF = spayed female.

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TABLE 6—Nine Canine Cutaneous Squamous Papillomas

Dog No.	Breed	Sex	Age (yr.)	Site	Size (cm.)	Characteristics
4989	.....	....	....	Hip	6.0 x 6.0	Many pearls.
52191	Bassett Hound	M	2	Stifle	....	Duration 6 mo.
5461	.....	....	8	Back	6.0 x 3.0	Pearls, inclusion cyst.
54670	Boston Terrier	....	7	Leg	0.5 x 0.5	Pedunculated, pearls.
551294	Poodle	M	12	.....	0.5 x 0.5	Pearls, lymphocytes.
551316	Terrier	....	5	Back	0.5 x 0.5	Pearls, inclusion cyst.
56817	Norwegian Elkhound	M	3	Neck	2.0 x 1.0	Lymphocytes, fibrosis.
A36	Fox Terrier	F	8	Neck	2.0 x 1.0	Encapsulated.
A54	Collie	M	8	Back	2.0 x 1.0	Pearls, strands.

and elongated rete ridges, possibly indicating precancerous changes.

All degrees of differentiation were present, from a very mature squamous cell carcinoma to a highly anaplastic carcinoma. The former consisted of well-keratinized cells, often arranged concentrically around flakes of conified cells, a picture customarily designated as onion ring or pearl formation. In the anaplastic type, only occasional areas of keratinization were evident. The cells were small and had round, hyperchromatic nuclei with numerous mitotic figures. Invasion of tumor cells into small veins and lymphatics was occasionally seen (fig. 17).

A grade designation of each tumor was applied in table 5 as an abbreviated method of indicating their degree of anaplasia. The grading was done on a morphologic basis, and the follow-up studies were too incomplete to permit conclusions as to the relationship between grading and biological behavior. The grading, according to Broders' classification, [32] was used with the numbers from 1 to 4, indicating increasing degrees of anaplasia.*

Summary and Conclusions.—Thirty-one squamous carcinomas of the canine skin were described in this study. Seventeen had local invasion and one had lymph node metastasis. The tumors occurred in old dogs of both sexes and without any breed predominance. The localization throughout the skin differed from that of the human squamous carcinoma which occurs most frequently in areas not protected by clothing.

SQUAMOUS PAPILLOMA

Review of Literature.—Mulligan,[35] in 1949, described the pathology of seven squamous papillomas occurring among 283 cutaneous epithelial neoplasms of the dog. The feet were a frequent site for this tumor which, in this location, was considered to be a precancerous lesion.

Gross Appearance.—The nine squamous papillomas in our collection measured from 0.5 by 0.5 cm. to 6.0 by 6.0 cm. No particular age, breed, sex, or site incidence could be established on the basis of the cases available (table 6). The papillomas encountered here were of unifocal occurrence and appeared as superficial, irregular, hard nodules with a rough and brittle surface.

Microscopic Appearance.—Growth from the hyperplastic epidermis of solid, wide strands of prickle cells was a characteristic finding. The strands often gave the impression of being exaggerated rete ridges with an increased growth tendency. The centers of the strands contained large cells in progressive stages of keratinization. Concentric arrangement of the cornified cells (so-called onion or pearl formation) was frequent (fig. 18). Fibrosis and infiltration with lymphocytes and plasma cells was seen in the connective tissue surrounding the strands. The epidermis covering a papilloma was frequently missing and the defect was filled with inflammatory cells and keratin debris. In dogs 5461 and 551316, the papilloma originated in the wall of an epidermal inclusion cyst. At the time of surgical removal, the cyst lumen was partially filled with papilloma tissue. Evidence of deeper invasion, metastasis, and recurrence was not detected.

Discussion.—The squamous papilloma is a benign epidermal neoplasm consisting of tumorous masses of mature and keratinizing epithelial cells derived from the stratum malpighii. It must be differentiated from two other types of papillomas occurring in the canine skin, the infectious papilloma and the fibrous papilloma, also called fibroepithelioma. Infectious papillomatosis is primarily a disease of the oral cavity and lips of the immature dog, and occurs rarely

* Grade of squamous carcinoma | 1 | 2 | 3 | 4
Percentage of differentiated cells | 75–100 | 50–75 | 25–50 | 0–25

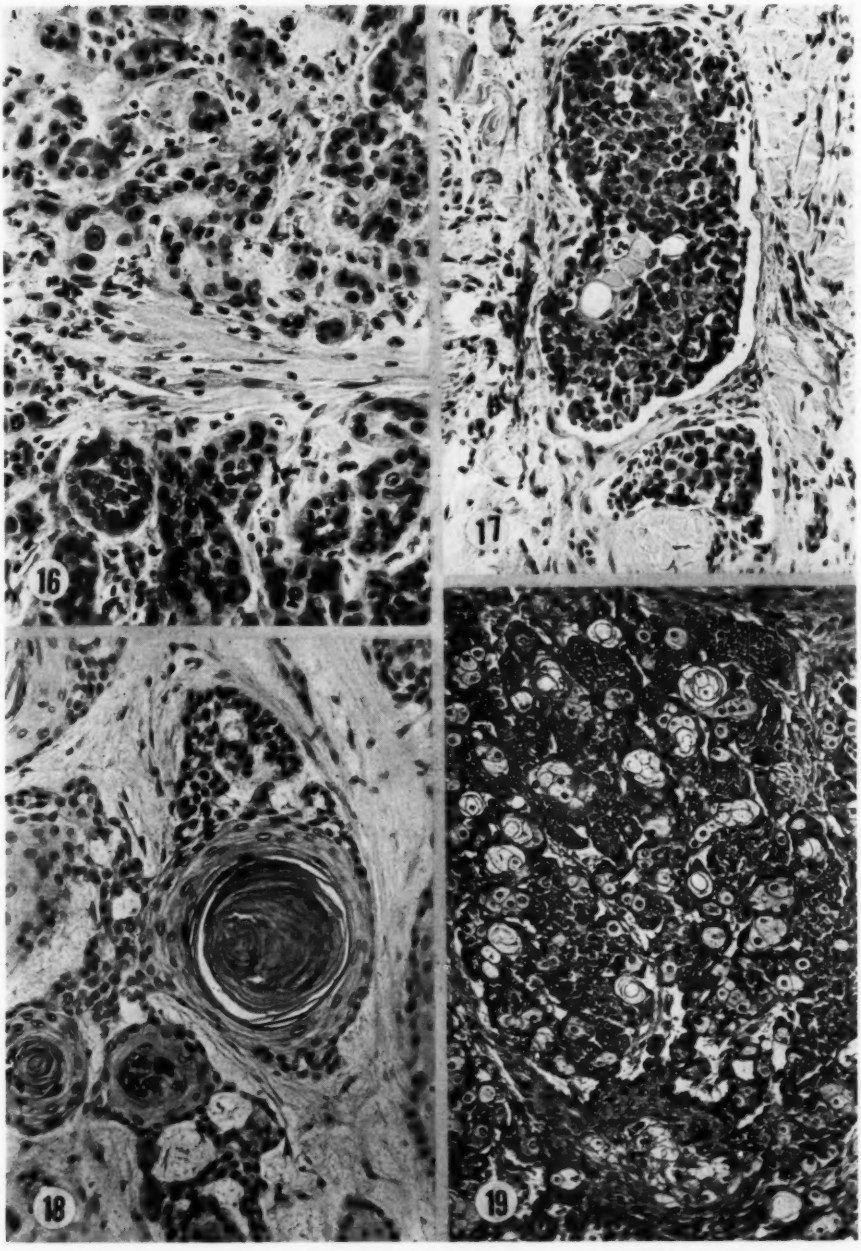

Fig. 16—Squamous cell carcinoma (5530), grade III malignancy, invading the collagen fibers of the corium. Anaplasia and many mitoses are present. H & E stain; x 470.

Fig. 17—Squmous cell carcinoma (B1323) of grade III malignancy illustrating invasion into lymphatic vesels of the corium. H & E stain; x 300.

Fig. 18—Squamous papilloma (551316) with marked keratinization and concentric lamination. The surrounding corium is loose and edematous. H & E stain; x 330.

Fig. 19—Sebaceous carcinoma (B1140) with invasion of the corium with solid lobules of basaloid cells with frequent sebaceous transition. H & E stain; x 170.

TABLE 7—Cutaneous Epithelial Neoplasms Encountered During 7,489 Pathologic Examinations of Dogs in a Ten-Year Period

Neoplasm	No. of dogs
Apocrine sweat gland tumor	14
Basal cell tumor	33
Trichoepithelioma	7
Calcifying epithelioma	5
Squamous cell carcinoma	31
Squamous papilloma	9
Sebaceous gland tumor	54
Total	153

as a solitary nodule. The fibrous papilloma is a true fibroma or a fibrous tissue proliferation covered by hyperplastic epidermis. The epidermis does not take an active part in the neoplastic process.

The differential diagnosis between a grade 1 squamous carcinoma and a squamous papilloma was occasionally difficult. In this study, two features were considered essential for distinguishing a papilloma from a squamous carcinoma: (1) a superficial location and (2) mature, highly keratinized, noninvasive growth.

Summary and Conclusions.—Nine squamous papillomas were found, ranging in size from 0.5 by 0.5 cm. to 6.0 by 6.0 cm., without any specific age, breed, sex, and site incidence. Superficial location, the absence of invasion, recurrence, and metastasis were important characteristics of this tumor.

V. Sebaceous Gland Tumors

The sebaceous glands of the canine skin are particularly large in the wire-haired and short-haired breeds, reaching their largest number and size in areas surrounding the mucocutaneous orifices. Disturbance of growth of these glands with the occurrence of seborrhea, hyperplasia, and tumor formation is of great clinical importance in the dog.

Review of Literature.—Livsey,[30] in 1906, described an ulcerating sebaceous carcinoma in the flank of an 11-year-old dog.

Cohrs,[6] in 1949, stated that solitary and multiple sebaceous tumors were frequent in the skin of dogs, particularly that of the head. Mulligan,[35] in 1949, found 45 sebaceous adenomas and 15 sebaceous carcinomas among 283 cutaneous epithelial neoplasms. Multiplicity and a frequent localization on the head were mentioned.

Head,[18] in 1953, diagnosed 55 sebaceous tumors among 709 canine neoplasms. Multiple occurrence was frequently seen in aged male dogs. No evidence of recurrence or metastases was observed.

Smith and Jones,[43] in 1957, mentioned the close resemblance of sebaceous adenomas to normal sebaceous glands and to basal cell tumors.

Gross Appearance.—Fifty-four canine sebaceous gland tumors were studied. In the majority of dogs, ulcerations were present, either grossly or microscopically. The tumors ranged in size from 0.3 by 0.2 cm. to 9.0 by 7.0 cm. All neoplasms larger than 3.0 by 2.0 cm. were histologically proven to be carcinomas. Of the involved dogs of known breed, 21 (42%) were Cocker Spaniels, a breed comprising only 19 per cent of the dog population in this area in 1956–1957.[38] Eleven dogs of the small terrier breeds were involved. There was no predominance of either sex, with 26 males and 21 females represented. The ages ranged from 6 to 16 years with an average age of 10 years. Sixteen of 49 tumors in which the site was recorded were located on the head, with the two most frequent sites being the eyelid and external ear. Multiple tumors were present in 11 dogs, and they were all found to be adenomas. One carcinoma recurred six months after removal. Metastases were not detected.

Microscopic Appearance.—Sebaceous adenomas were diagnosed in 29 dogs and sebaceous carcinomas in 25 dogs. The adenomas consisted of lobulated masses of immature sebaceous glands located in the dermis. The overlying epidermis was frequently ulcerated, and there was evidence of inflammation, probably due to infection. The mature portions of the sebaceous adenomas were indistinguishable from normal sebaceous glands. The immature structures appeared as solid lobules of basaloid reserve cells with ovoid hyperchromatic nuclei and dark cytoplasms. Cells in the central portions of such lobules frequently had undergone sebaceous transition. This appeared as a distention of the cells with a foamy vacuolization of the cytoplasm and nuclear pyknosis.

The sebaceous carcinomas were seen as solid lobules and plump strands invading the dermis and subcutis (fig. 19). They were composed of densely packed basaloid cells with hyperchromatic nuclei and basophilic cytoplasm. The nuclei had no polarity, contained from one to three small nucleoli, and were frequently in mitotic division. The cytoplasm was dark, poorly outlined, and present only in a small amount. Occa-

sional cells in the central portion of the lobules were undergoing sebaceous transition identical to the process described for the adenomas.

Summary and Conclusions. The 54 canine sebaceous tumors studied included 25 carcinomas and 29 adenomas; eleven of the latter were multiple. One sebaceous carcinoma recurred six months after surgical removal. The average age of the dogs was 10 years. The Cocker Spaniel had some breed predisposition to this tumor. One third of the tumors occurred on the head, with the eyelid and external ear being the most frequent site.

The multiplicity of the sebaceous adenoma is a unique feature that has not been encountered in any of the other cutaneous epithelial tumors of the dog.

Summary and Conclusions

Pathologic examination of 7,489 canine skin specimens submitted to the Department of Veterinary Pathology of the Ohio State University in the ten-year period from 1949 to 1959 revealed epithelial neoplasms in 153 dogs (table 7). Viral papillomatosis and tumors originating in the special glandular structures, the mammary and perianal glands, were not included in this study. The incidence of cutaneous epithelial tumors in this canine material was 2 per cent.

Fourteen apocrine sweat gland tumors were found, three of which were diagnosed as mixed apocrine sweat gland tumors with cartilage formation. No report of canine mixed sweat gland tumors was found in the literature.

Of 33 basal cell tumors encountered, 80 per cent were in the skin of the head and neck, a site incidence comparable to that of man. Two different patterns of the basal cell tumor were recognized, the ribbon and the solid types. The former has been classified by some authors as sweat gland tumor. The solid type was the most aggressive and recurred in 3 dogs.

Seven calcifying epitheliomas and five trichoepitheliomas were present. The calcified epithelioma appeared to be a late stage of the trichoepithelioma that had undergone mummification, central necrosis, and mineralization. It was also pointed out that certain cutaneous lesions currently classified as calcinosis circumscripta represent calcifying epitheliomas that are totally mineralized. The trichoepitheliomas and calcifying epitheliomas were consistently benign in clinical behavior.

Squamous cell carcinomas were present in 31 dogs. This was the only type of epithelial tumor in which unequivocal evidence of metastasis was demonstrated in this study.

Sebaceous gland tumors occurred in 54 dogs and were most prevalent of all cutaneous epithelial neoplasms. The frequency of this tumor was further augmented by its multiplicity, as 11 dogs had multiple sebaceous gland adenomas. None of the other cutaneous epithelial tumors were found to be of multiple occurrence.

References

[1] Albertini, A.: Histologische Geschwulstdiagnostik. Georg Thieme Verlag, Stuttgart, Germany, 1955.

[2] Allen, A. C.: The Skin. C. V. Mosby, St. Louis, 1954.

[3] Allen, A. C.: So-Called Mixed Tumors of Mammary Glands of Dog and Man. Arch. Path., 29, (1940): 589–624.

[4] Anderson, N. P.: Hidradenoma of the Vulva. Arch. Dermat. & Syph., 62, (1950): 873–892.

[5] Boucek, Z.: Mitteilungen über 35 histologisch untersuchte Tiergeschwulste. Arch. wissensch. u. prakt. Thierh., 32, (1906): 585. (Quoted by Folger.[14])

[6] Cohrs, P.: Lehrbuch der speziellen pathologischen Anatomie der Haustiere. G. Fischer, Jena, Germany, 1954.

[7] Cotchin, E.: Further Observations on Neoplasms in Dogs, with Particular Reference to Site of Origin and Malignancy. Brit. Vet. J., 110, (1954): 218–230.

[8] Cotchin, E.: Neoplasms of the Domesticated Mammals. Commonwealth Agricultural Bureau, England, 1956.

[9] Cotchin, E.: Mammary Neoplasms of the Bitch. J. Comp. Path. & Therap., 68, (1958): 1–22.

[10] Creed, R. F. S.: Histology of Mammalian Skin, with Special Reference to the Dog and Cat. Vet. Rec., 70, (1958): 1–7.

[11] Eller, J. J., and Eller, W. D.: Tumors of the Skin. Lea & Febiger, Philadelphia, 1951.

[12] Erichsen, S.: A Histochemical Study on Mixed Tumours of the Canine Mammary Gland. Acta path. et microbiol. scandinav., 36, (1955): 481–489.

[13] Feldman, W. H.: Neoplasms of Domesticated Animals. W. B. Saunders Co., Philadelphia, 1932.

[14] Folger, A. F.: Geschwulste bei Tieren. Ergebnisse d. allg. Path. u. path. Anat., 18, (1917): 372–676.

[15] Foot, N. C.: Adnexal Carcinomas of the Skin. Am. J. Path., 23, (1947): 1–27.

[16] Gates, O., Warren, S., and Warvi, W. N.: Tumors of Sweat Glands. Am. J. Path., 19, (1943): 591–631.

[17] Groth, W.: Basaliome der Haut beim Hund und Kaninchen. Ztschr. Krebsforsch., 60, (1955): 361–372.

[18] Head, K. W.: Skin Diseases, Neoplastic Diseases. Vet. Rec., 65, (1953): 926–929.

[19] Highman, B., and Ogden, G. E.: Calcified Epithelioma. Arch. Path., 37, (1944): 169–174.

[20] Hulett, R. M.: Trichomatrioma. Arch. Dermat. & Syph., 77, (1958): 285–296.

[21] Innes, J. R. M.: Malignant Diseases of Domesticated Mammals. Cancer, 3, (1957): 73–115.

[22] Jackson, C.: The Incidence and Pathology of Tumors of Domesticated Animals in South Africa. Onderstepoort J. Vet. Sci. & Anim. Indust., 6, (1936): 11–460.

[23] King, L. S.: Mummified Epidermal Cysts (So-Called Calcified Epithelioma). Am. J. Path., 23, (1947): 29–37.

[24] Kretzschmar, C.: Zur Kalkgicht des Hundes und ihrer Beziehung zur chronischen Nephritis. Deutsche tierärztl. Wchnschr., 31, (1955): 320–322.

[25] Kuang-Yu, C.: Calcified Epithelioma of Skin. Am. J. Path., 9, (1933): 497–524.

XUM

[28] Kunze, A.: Über multiple Kalkeinlagerungen in der Unterhaut der Extremitäten des Hundes. Arch. f. wissensch. prakt. Thierheilk., 54, (1926): 462–478.
[27] Larsson, B.: Statistical Analyses of Cutaneous Tumors in Dogs. Nord. Vet.-med., 8 (1956): 130–139.
[28] Lennox, B.: The Superficial Hidradenomata. J. Path. & Bact., 67, (1954): 553–562.
[29] Lever, W. F.: Histopathology of the Skin. Lippincott Co., Philadelphia, 1949.
[30] Livsey, G. H.: A Case of Epidermal Carcinoma in the Dog. J. Comp. Path. & Therap., 19, (1906): 43. (Quoted by Folger.[14])
[31] Lovell, J. E., and Getty, R.: The Hair Follicle, Epidermis and Skin Glands of the Dog. Am. J. Vet. Res., 18, (Oct., 1957): 873–885.
[32] Lund, H. Z.: Tumors of the Skin. Fascicle 2. Armed Forces Inst. Path., Washington, 1957.
[33] McDonald, J. R.: Apocrine Sweat Gland Carcinoma of the Vulva. Am. J. Clin. Path., 11, (1941): 890–897.
[34] Moulton, J.: Histological Classification of Canine Mammary Tumors. Cornell Vet., 44, (1954): 168–180.
[35] Mulligan, R. M.: Neoplasms of the Dog. Williams & Wilkins Co., Baltimore, 1949.
[36] Murray, J. A.: The Zoological Distribution of Cancer. Imp. Canc. Res. Fund, 41, (1908): 41–68.
[37] Nielsen, S. W.: Glands of the Canine Skin. Am. J. Vet. Res., 14, (July, 1953): 448–454.
[38] Nielsen, S. W., and Cole, C. R.: Canine Mastocytoma. A Report of One Hundred Cases. Am. J. Vet. Res., 19, (April, 1958): 417–432.
[39] Novak, E., and Stevenson, R. R.: Sweat Gland Tumors of the Vulva, Benign (Hidradenoma) and Malignant (Adenocarcinoma). Am. J. Obst. & Gynec., 1, (1945): 641–659.
[40] Pallaske, G.: Pathologische Histologie. Gustav Fischer, Jena, Germany, 1955.
[41] Pinkus, H.: Premalignant Fibroepithelial Tumors of Skin. Arch. Dermat. & Syph., 67, (1953): 598–615.
[42] Sandersleben, J.: Über epitheliale Neubildungen in der Haut des Hundes. Berl. u. Münchener tierärztl. Wchnschr., 71, (1958): 287–290.
[43] Smith, H. A., and Jones, T. C.: Veterinary Pathology. Lea & Febiger, Philadelphia, 1957.
[44] Speed, J. G.: Sweat Glands of the Dog. Vet. J., 97, (1941): 252–256.
[45] Stout, A. P., and Cooley, S. G. E.: Carcinoma of Sweat Glands. Cancer, 4, (1951): 521–536.
[46] Swerdlow, M.: Histogenesis of Basal-Cell Carcinoma. Arch. Dermat. & Syph., 78, (1958): 563–570.
[47] Teloh, H. A.: Apocrine Adenoma of the Anus. Cancer, 7, (1954): 367–372.
[48] Tilden, I. L.: Mummifying Epithelioma. Arch. Dermat. & Syph., 66, (1952): 728–737.
[49] Turhan, B., and Krainer, L.: Bemerkungen über die sogenannten verkalkenden Epitheliome der Haut und ihre Genese. Dermatologica, 85, (1942): 73–89.
[50] Warvi, W. N., and Gates, O.: Epithelial Cysts and Cystic Tumors of Skin. Am. J. Path. 19, (1943): 765–781.
[51] Webb, A. J., and Calhoun, M. L.: The Microscopic Anatomy of the Skin of Mongrel Dogs. Am. J. Vet. Res., 15, (April, 1954): 274–280.
[52] Willis, R. A.: Pathology of Tumors. Butterworth, London, England, 1948.

SUMMARIO IN INTERLINGUA

Neoplasma Epithelial Cutanee in Canes—Un Reporto de 153 Casos

Le examines pathologic de 7.489 specimens de pelle canin, submittite al Departimento de Pathologia Veterinari del Universitate Statal de Ohio in le curso del decennio ab 1949 ad 1959, resultava in le constatation de 153 casos de neoplasma epithelial. Papillomatosis virusal e tumores a origine in le structuras glandular special del mammas e del glandulas perianal non esseva includite in le presente studio. Assi le incidentia de tumores epithelial cutanee in iste casuistica esseva 2 pro cento.

Esseva trovate 14 casos de tumor de apocrin glandulas sudorifere. Tres de istos esseva diagnosticate como mixte tumores de apocrin glandulas sudorifere con formation de cartilagine. Nulle reporto de mixte tumores de canin glandulas sudorifere esseva trovate in le litteratura.

Inter le 33 casos de tumores de cellulas basal, 80 pro cento esseva in le pelle del capite e del collo. Iste incidentia topographic es comparabile a illo reportate pro humanos. Duo differente configurationes de tumor de cellulas basal esseva recognoscite, le typo bandate e le typo solide. Le prime de iste duo typos es classificate per certe autores como un tumor de glandula sudorifere. Le typo solide esseva le plus aggressive, con recurrentias in 3 canes.

Septe epitheliomas calcificante e cinque trichoepitheliomas esseva representate. Le forma calcificate de epithelioma pareva esser un stadio tardive de trichoepithelioma modificate per mummification, necrosis central, e mineralisation. Es etiam signalate que certe lesiones cutanee que es currentemente classificate como calcinosis circumscripte es de facto epitheliomas calcificante que es completemente mineralisate. Le trichoepitheliomas e le epitheliomas calcificante esseva uniformemente benigne in lor comportamento clinic.

Carcinomas a cellulas squamose esseva presente in 31 canes. Isto esseva le sol typo de tumor epithelial pro le qual evidentia inequivoc de metastase esseva trovate in le presente studio.

Tumores de glandula sebacee occurreva in 54 canes. Illos esseva le plus frequente de omne le typos de neoplasma epithelial cutanee. Le frequentia de iste tumor esseva augmentate additionalmente per le facto de su multiplicitate, i.e.: 11 canes habeva multiple adenomas de glandula sebacee. Nulle del altere cutanee tumores epithelial esseva representate in occurrentias multiple.

Evaluation of the Bromsulfophthalein Liver Function Test in the Dog

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SINCE ITS DEVELOPMENT in 1925,[23] the bromsulfophthalein (BSP) liver function test, through modification and improvement, has become a standard procedure in human medicine. It has not found wide application in veterinary practice because, as Coffin [6] states, the economic considerations possibly limit its use. There is only scant accumulation of data from clinical employment of the test in dogs, although numerous investigators have utilized it in studying the action of specific agents on the liver or in comparing it with the other tests for hepatic dysfunction. It is a useful test in the laboratory for following experimentally induced hepatic disease and for drug toxicopathologic investigations.

The purpose of this study was to evaluate critically the BSP liver function test in the dog by determining the degree of correlation between percentage of dye retention and the type and extent of hepatic damage. Attention was given to the effect of fever and of lesions in other organs and tissues, especially the reticuloendothelial system, on the retention of dye.

REVIEW OF LITERATURE

The superiority of the BSP liver function test over other determinations for the early detection of hepatic dysfunction in animals has been demonstrated by Drill and Ivy (dog),[8] Casals and Olitsky (mouse),[5] Svirbely *et al.* (dog),[24] Hoerlein and Green (dog),[13] Gornall and Bardawell (dog),[11] and Linder *et al.* (rat).[17]

The fate of the dye after injection has been investigated extensively. Transient elevated retention of BSP following blockage of the reticuloendothelial system was reported by Klein and Levinson (dog),[14] and Mills and Dragstedt (dog).[19, 20] The latter investigators produced no reduction in dye clearance with bile ligation, but they enhanced retention by administration of a cholegogue. Cantarow and Wirtz (dog)[4] determined that 50 to 83 per cent of BSP was excreted in the bile in the first hour and 67 to 100 per cent within two hours. Cohn *et al.* (dog)[7] found 31 to 65 per cent was recoverable in the bile; peripheral tissue in the partially eviscerated animal was capable of removing the dye 25 to 30 per cent as rapidly as an intact one. In the liver perfusion experiments of Bauer and Pessotti (rat),[1] uptake of dye was independent of metabolic processes; whereas, damage to the vascular bed caused a reduction. They later reported 77 to 89 per cent of the dye in the blood stream was extracted by the liver (dog).[2] Bauer *et al.*[3] made nearly quantitative recovery of S^{35}-tagged BSP from the liver, blood, and bile of dogs. Working with surgically altered dogs, Wirtz *et al.*[25] hypothesized that impaired capacity to excrete BSP is first manifested by delay in transfer from hepatic cells to lumina of biliary canuliculi. As functional damage increases, the hepatic cells become saturated with dye and lose their ability to remove it from the blood. Giges *et al.*[10] recovered 30 to 50 per cent of the injected dye in the urine of dogs. Pratt *et al.*[22] reported that loss of BSP in the urine and restoration of dye to the blood stream by intestinal resorption or lymphatic return was insignificant in their infused and cannulated dogs.

Differences in method have resulted in a wide variation of dye retention values in normal and hepatically damaged dogs. Drill and Ivy [8] found that normal retention was between 2 and 12 per cent in 30-minute serum samples from dogs given injections of dye at 5 mg. per kilogram of body weight (using 2 mg./kg. standards). They regarded retentions over 15 per cent as abnormal and recorded values as high as 250 per cent (with the 2 mg./kg. standard) in dogs with hepatic damage from carbon tetrachloride. Using the spectrophotometric method in pups given 7 mg./kg. of the dye (yielded samples at 8 minutes), McKibbin *et al.*[18] accepted 3 to 8 μg. per milliliter as normal and found values of 12 to 42 μg. in dogs with fatty metamorphosis produced by choline deficiency. Svirbely *et al.*,[24] using the spectrophotometric technique on samples from dogs given 5 mg./kg. of BSP and sampled at five and 30 minutes, obtained average normal retentions of 2.5 to 13.2 per cent (average, 6.2 per cent). In dogs with xylidine-damaged livers, they recorded values as high as 65 per cent and, in those with fatty metamorphosis, values of

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14.1 per cent when the control reading was 4.8 per cent. Noyan[21] appraised the value of the BSP test by comparing the results in 50 normal dogs with those with miscellaneous disturbances. The technique entailed injection of 5 mg./kg. of the dye and sampling of the blood in 30 minutes. Percentage of dye retention was determined in a Rouy-Leitz photometer. Normal dogs always had retention below 10 per cent with the majority at 5 per cent. The highest retentions (64%) resulted from hepatic damage in the form of fatty metamorphosis caused by carbon tetrachloride. In 7 of 24 dogs afflicted with distemper, retentions up to 10 per cent were recorded, and, in serums from pups inoculated with fox encephalitis virus, as high as 27 per cent of the dye was retained. He found false positives were attributable to fever, heart failure, ascites, obstructed bile ducts, and parenterally injected dyes. He considered the test more reliable in acute than in chronic liver disorders and valuable in hepatitis and fatty degeneration but questionable in cirrhosis.

Hoerlein and Greene,[13] using the method of Rosenthal and White[23] which utilizes the comparator block, but substituting 5 mg./kg. of the dye instead of the 2 mg./kg. dosage, concluded that absence of retention at 30 minutes was normal. In 50 clinical cases, 22 had abnormal values of 5 to 10 per cent and 3 had 100 per cent retention. Of 16 dogs with miscellaneous clinical cases, 2 of 3 animals with distemper had 10 per cent retentions. They concluded that the test was of value in confirming early hepatic dysfunction, such as cirrhosis, fatty hepatosis, and other liver conditions in which the animal does not have abnormal bilirubinemia; they considered the test quantitative in indicating the amount of liver damage.

Lamson and Wing[16] demonstrated that as little as 0.3 cc. of carbon tetrachloride produced early cirrhosis in dogs if given every other day for 15 weeks. They had previously reported[15] that threshold doses of 0.5 to 1.0 cc./kg. produced central necrosis of the liver.

MATERIALS AND METHODS

The BPS used in these studies was the commercial* 5 per cent (50 mg./ml.) aqueous solution of sulfobromophthalein sodium, U.S.P. (phenoltetrabromphthalein-disodiumsulfonate). The undiluted commercial solution was administered intravenously at 5 mg./kg. of body weight to all dogs. A Beckman Model B spectrophotometer was used to determine the dye content of the serum. Hepler's[12] procedure, which is essentially that of Gaebler,[9] was used for estimating BSP retention. Estimations of dye content were made on blood samples taken at 45 minutes.

The 85 dogs in these studies ranged from nondescript dogs of mixed breeding to purebreds (beagles) and were from a variety of sources. The majority of the beagles were from the Upjohn colony or had been secured for the colony from local breeders; complete histories of these dogs were available. Dogs of doubtful breeding, recognizable crossbreds, and a number of probable purebreds were from dog pounds within a limited geographical radius from the laboratory; histories were not obtainable for

* Produced by Hynson, Westcott and Dunning, Inc., Baltimore, Md.

TABLE 1—Agents and Methods Used on 85 Dogs for Inducing or Evaluating Hepatic Injury

No. of dogs	Dog No. (incl.)	Sex	Agent	Dosage	Duration of administration (days)
14	5921-23, 6072-74, 6188-90, 6249-51, 6468-69	9 M, 5 F	None	.....	.....
5	6664, 6796, 7071-73	2 M, 3 F	Carbon tetrachloride	0.25, 0.4, and 0.5 cc.	20, 60, 117
6	5924-29	6 M	Drug A (streptovaricin)	30 and 100 mg./kg.	64
8	6075-82	3 M, 5 F	Drug B (tolbutamide, Na salt)	30, 100, and 160 mg./kg.	180
9	6191-99	5 M, 4 F	Drug C (dipropyl-allyloxy-benzoic acid)	30, 100, and 300 mg./kg.	30
6	6241, 6243-47	1 M, 5 F	Drug D (pyrazolidin-3-one, 1-methyl-5-phenyl)	30 and 60 mg./kg.	28, 35
6	6463-66, 6470-71	1 M, 5 F	Drug E (methyl reserpate)	1, 15, and 50 mg./kg.	21, 28
20	6334-(1-3, 543) 6337-(4-10) 6338-(a-c) 6344-(1-2) 6345-(3-5) 6443-6	15 M, 5 F	Canine distemper complex	.....	.....
6	6492, 6681, 6697, 6760-62	3 M, 3 F	Infectious canine hepatitis virus	.....	2
5	6446-(1-5)	1 M, 4 F	None (fever)	.....	.....

these. The animals ranged in age from several months to aged (average, 2 yr.); both sexes were represented.

Of the 85 dogs, 14 were normal controls; 5 had been given carbon tetrachloride; 35 had been given other drugs; 20 had clinical signs of the distemper complex; 6 had been inoculated with canine infectious hepatitis virus; and 5 were pyretic only (table 1). The drugs used in the study were: streptovaricin (drug A); tolbutamide, sodium salt (drug B); dipropyl-allyloxybenzoic acid (drug C); pyrazolidin-3-1, 1-methyl-5-phenyl (drug D); and methyl reserpate (drug E).

The normal control dogs (9 males, 5 females) were purebred colony beagles which had served as controls for toxicopathologic studies. Two males and 3 females of mixed breeding had been given carbon tetrachloride. Beagles from the colony were employed in all the drug studies: 6 males had been given drug A; 3 males and 5 females, drug B; 5 males and 4 females, drug C; 1 male and 5 females, drug D; and 1 male and 5 females, drug E. Fifteen male dogs (5 were colony beagles and the remainder were of pound origin) and 5 females (all of pound origin), manifesting signs of the canine distemper complex, were subjects of this study. Presumptive diagnosis was made from physical examination. Six very immature pound dogs (3 males, 3 females) were inoculated subcutaneously on two successive days with 0.5 ml. of infectious canine hepatitis virus.* This virus had proved its pathogenicity in susceptible animals. Five immature pound dogs (1 male and 4 females) of mixed breeding, secured for the experimental canine infectious hepatitis study, were found on preliminary physical examination to have elevated body temperatures but no other physical signs of disease. The liver function test and euthanasia were performed on the same day as the physical examination.

All of the animals were killed by electrocution. Partial exsanguination immediately followed. A general necropsy was performed on each dog and blocks of tissue were removed, fixed in 10 per cent formalin solution, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin. Fat-stained sections, formalin-fixed, were prepared either by the freezing method or were embedded in Carbowax and stained with Sudan IV or oil red O.

Results

The results are summarized (table 2). Retentions of 0.8 per cent *or less* are reported as 0.8 per cent.

Dye retention of 0.8 per cent was recorded for 13 and 1.5 per cent for 1 of the 14 control dogs. On necropsy, however, 8 of the 14 had grossly discernible lesions in various organs and tissues. The livers, both grossly and microscopically, were lesionless. On histopathologic examination, various other organs were seen to have a wide variety of spontaneous lesions.

All the dogs given CCl_4 lost over a kilogram of body weight during the test period. The BSP retention was 0.8 to 34.0 per cent. Dog 6796, with a dye retention of 0.8 per cent, had residual hepatic damage consisting of hepatocellular swelling, mild cirrhotic change, and centrolobular bile stasis. Severe hepatocellular degeneration and disturbed circulation were primary histologic alterations in the liver of dog 6664 which had a 21.8 per cent retention. In dogs 7071, 7072, and 7073, the dye retention of 34.0, 29.8 and 23.6 per cent, respectively, varied inversely to the degree of cirrhosis encountered and directly with acute liver damage.

In dogs given drug A, BSP retention was 1.5 to 9.0 per cent. Retentions in dogs 5924, 5925, and 5926 were 2.2, 2.2, and 4.8 per cent, respectively, but no abnormalities were found at gross and histologic examination. Dog 5927, with 3.6 per cent retention, had no gross or microscopic hepatic lesions. Dog 5928 retained 1.5 per cent of dye in the serum and had focal lymphocytic infiltration or proliferation in the hepatic parenchyma. With a 9.0 per cent retention, dog 5929 had early, acute, purulent hepatitis.

With drug B, retentions of 0.8, 0.8, 1.5, 2.2, and 1.8 per cent, without hepatic alteration, were recorded for dogs 6075, 6076, 6077, 6078, and 6080. Dog 6079 had diffuse hepatocellular vacuolization and retained 4.3 per cent of the dye. Dogs 6081 and 6082 had values of 4.3 and 1.5 per cent for the function tests; mild, diffuse, subacute hepatitis was detected microscopically in the livers of both dogs. Gross pathologic hepatic change was seen in dog 6081 but not in dog 6082.

With drug C, the livers were essentially normal macroscopically and microscopically in dogs 6192, 6193, 6194, 6195, 6196, 6197, and 6199; dye retention was 0.8 per cent in each. Dog 6194, with 0.8 per cent retention, had an allergic hepatic response histologically, characteristic of parasitic injury. Scattered inflammatory foci and pigment accumulation were observed in the liver of dog 6191 which retained 2.2 per cent dye. Dog 6198 retained a serum concentration of BSP of 41.2 per

* CIHV, produced by the Virology Section, the Upjohn Company, Kalamazoo, Mich.

TABLE 2—Summary of Hepatic Histopathologic Findings and Bromsulfophthalein Retention

Dog No.	Sex	Agent*	BSP ret.	Gross evidence	Hepatic histopathology
5921	M	N	0.8	....	.....
5922	M	N	0.8	....	.....
5923	M	N	0.8	....	.....
6072	M	N	0.8	....	.....
6073	F	N	0.8	....	.....
6074	F	N	0.8	....	.....
6188	M	N	0.8	....	.....
6189	M	N	0.8	....	.....
6190	F	N	0.8	....	.....
6249	F	N	0.8	....	.....
6250	F	N	1.5	....	.....
6251	M	N	0.8	....	.....
6468	M	N	0.8	....	.....
6469	M	N	0.8	....	.....
6664	F	CT	21.8	X	Centrolobular degeneration; infrequent necrosis; severe centrolobular fatty metamorphosis.
6796	M	CT	0.8	....	Hepatocellular swelling; mild fibrosis; bile stasis.
7071	M	CT	34.0	X	Severe degeneration; hepatocellular necrosis; hemorrhage; mild perilobular fibrosis; mild bile duct proliferation; mild regeneration.
7072	F	CT	29.8	X	Severe degeneration; centrolobular hemorrhage; pigment accumulation; mild perilobular and periportal fibrosis.
7073	F	CT	23.6	X	Hepatocellular degeneration and regeneration; moderate peripherolobular fibrosis and hemorrhage; mild bile duct proliferation and pigment accumulation.
5924	M	A	2.2	....	.....
5925	M	A	2.2	....	.....
5926	M	A	4.8	....	.....
5927	M	A	3.6	....	.....
5928	M	A	1.5	....	Scattered foci of lymphocytes.
5929	M	A	9.0	....	Central vein perivasculitis.
6075	M	B	0.8	X	**
6076	M	B	0.8	X	**
6077	F	B	1.5	....	.....
6078	M	B	2.2	....	.....
6079	F	B	4.3	....	Hepatocellular vacuolization (not fat).
6080	F	B	1.8	....	.....
6081	F	B	4.3	X	Moderate subacute hepatitis; peripherolobular fatty metamorphosis.
6082	F	B	1.5	....	Essentially similar to that of dog 6081.
6191	F	C	2.2	....	Scattered inflammatory foci and pigment.
6192	M	C	0.8	....	.....
6193	M	C	0.8	....	.....
6194	F	C	0.8	....	Focal allergic reaction (parasitic).
6195	F	C	0.8	....	.....
6196	M	C	0.8	....	.....
6197	M	C	0.8	....	.....
6198	F	C	41.2	X	Centrolobular congestion and hepatocellular degeneration.
6199	M	C	0.8	....	.....
6245	F	D	16.2	X	Moderate centrolobular degeneration; marked centrolobular pigment accumulation.
6246	F	D	9.8	X	Moderate centrolobular accumulation of pigment; centrolobular endothelial hypertrophy and mild hemorrhage; mild fatty metamorphosis.
6247	F	D	6.2	X	Mild centrolobular accumulation of pigment and fatty metamorphosis; bile duct epithelial hypertrophy.
6243	M	D	21.8	X	Centrolobular: hepatocellular degeneration and necrosis, pigment accumulation, stromal degeneration, and marked fatty metamorphosis.
6244	F	D	10.5	X	Centrolobular: pigment accumulation, marked hepatocellular degeneration, mild fatty metamorphosis.
6241	F	D	7.0	X	Centrolobular: moderate pigment accumulation, marked hepatocellular degeneration, and occasional necrotic foci; diffuse moderate fatty metamorphosis.
6470	F	E	0.8	....	Very mild focal subacute hepatitis.
6471	F	E	0.8	....	.....
6463	F	E	0.8	....	.....
6464	F	E	0.8	....	.....
6465	M	E	0.8	....	.....
6466	F	E	0.8	....	.....
6334-1	F	CDC	0.8	....	Very mild, early degenerative changes.
6334-2	M	CDC	2.5	X	Mild hepatocellular degeneration.

TABLE 2 (continued)—Summary of Hepatic Histopathologic Findings and Bromsulfophthalein Retention

Dog No.	Sex	Agent*	BSP ret.	Gross evidence	Hepatic histopathology
6334-3	M	CDC	4.8	X	Hepatocellular degeneration and centrolobular congestion.
6334-543	M	CDC	0.8	....	Mild hepatocellular degeneration.
6337-4	M	CDC	0.8	X	Mild passive congestion and hepatocellular degeneration.
6337-5	M	CDC	1.2	....	Mild hepatocellular degeneration.
6337-6	F	CDC	0.8	....	.....
6337-7	M	CDC	0.8	X	Mild to moderate, focal, acute to subacute hepatitis.
6337-8	F	CDC	6.2	....	Generalized hepatocellular degeneration.
6337-9	F	CDC	9.8	X	Marked focal acute hepatitis.
6337-10	M	CDC	5.9	X	Moderate vascular degeneration; moderate focal subacute to chronic hepatitis; larval granulomatosis.
6338-a	M	CDC	2.2	....	Mild focal, acute to subacute, hepatitis.
6338-b	M	CDC	0.8	X	Very mild focal subacute hepatitis.
6338-c	M	CDC	1.5	....	Very mild focal subacute hepatitis.
6344-1	F	CDC	1.8	X	**
6344-2	M	CDC	0.8	....	.....
6345-3	M	CDC	4.0	....	Moderate focal acute hepatitis.
6345-4	M	CDC	1.5	....	Very mild hepatocellular degeneration.
6345-5	M	CDC	0.8	....	.....
6443-6	M	CDC	2.2	X	Larval granulomatosis.
6492	F	CIH	5.9	X	Moderate hepatocellular degeneration; mild focal subacute hepatitis; nucleolar polychromatism.
6681	M	CIH	4.3	X	Very mild acute hepatitis; diffuse hepatocellular degeneration.
6760	F	CIH	0.8	X	**
6761	M	CIH	0.8	....	.....
6762	M	CIH	0.8	....	Moderate focal, subacute to chronic, hepatitis.
6697	F	CIH	12.0	X	Marked focal acute hepatitis.
6446-1	F	P	0.8	....	Larval granulomatosis.
6446-2	F	P	0.8	....	.....
6446-3	F	P	0.8	....	.....
6446-4	F	P	0.8	....	.....
6446-5	M	P	0.8	....	.....

* Key to agents: N = control; CT = carbon tetrachloride; A, B, C, D = Drugs A, B, C, D (*see* text); CDC = canine distemper complex; CIH = canine infectious hepatitis virus; P = pyrexia. ** Histopathologic examination did not confirm gross observations.

cent and had hepatic congestion and degeneration of hepatic cells at necropsy.

Hepatopathy of varying degrees occurred in all the dogs in the drug D evaluation. Likewise, elevated dye retentions were recorded for all the subjects. Hepatotoxic manifestations observed at necropsy consisted principally of alterations in the color of the organ and less consistently in changes in texture. Histologically, the lesions were characterized by pigment (probably hematogenous) accumulation and damage to the hepatic cells and vascular bed. In dog 6247 (6.2% retention), the tissue alterations were relatively mild; whereas, in dog 6243, the hepatic damage was marked, the pigment abundant, and blood clearance of the dye delayed, resulting in a 21.8 per cent retention. Extending the blood sampling time to an hour and 45 minutes resulted in a 7.0 per cent retention in dog 6241 which sustained severe hepatic damage from the drug.

The 6 dogs given drug E had BSP retentions of 0.8 per cent. With one exception, the livers of these dogs were free of abnormal changes. Microscopic examination of the liver of dog 6470 disclosed mild, focal, subacute hepatitis.

A definitive diagnosis of canine distemper based on the presence of intranuclear or intracytoplasmic inclusion bodies in tissues, was made in 6 of the 20 dogs (6337-4, 6337-6, 6337-9, 6337-10, 6338-c, and 6443-6). Eight dogs had BSP retentions of 0.8 per cent; retentions of 1.5 to 9.8 per cent were recorded for the other 12 dogs. In the former, three of the livers were lesionless, three had early degenerative changes, and two contained lesions of mild, focal, subacute hepatitis. In dogs with retentions between 0.8 and 3.9 per cent, degenerative changes predominated in the livers, but 1 dog had mild focal hepatitis and 1 had larval granulomatosis; both animals retained 2.2 per cent of dye. The livers of dogs having retentions of 4.0 per cent and over contained, principally, lesions indicative of focal hepatitis, centrolobular ne-

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crosis, or centrolobular hemorrhage and congestion.

The dogs inoculated with infectious canine hepatitis virus reflected the variability in reactions to the virus in the degree of dye retention and hepatic lesions. Dogs 6760, 6761, and 6762 retained 0.8 per cent, with no liver lesion in dogs 6760 and 6761; dog 6762 had a moderate, focal, subacute (bordering on chronic) hepatitis. The liver was yellowish grossly and there was a very mild acute focal hepatitis in dog 6681, which retained 4.3 per cent of the dye. A mild, focal, subacute hepatitis; moderate centrolobular fatty metamorphosis; and inexplicable tinctorial nuclear change (reversal of staining affinity or mauve nucleolar color) was observed, with a 5.9 per cent BSP retention, in dog 6492. The hepatic lesions in dog 6697, characterized macroscopically by irregular yellowish mottling and microscopically by disseminated acute focal hepatitis, caused a BSP retention of 12.0 per cent. Intranuclear inclusion bodies were not engendered by this virus.

The BSP retention in each of the 5 pyretic dogs was 0.8 per cent. The livers were essentially normal on macroscopic and microscopic examinations, with two exceptions: dog 6446-1 had mild, hepatic, larval granulomatosis and dog 6446-5 had mild, hepatocellular, cloudy swelling. The cause of fever could be determined with certainty in only 2 of the dogs (early bronchopneumonia in 1 and severe unilateral tonsillitis and lymphadenitis of the suprapharyngeal lymph node in the other). The lesions observed in dogs 6446-2, 6446-3, 6446-4, and 6446-5 suggest that they were in the prodromic stage of systemic infection, conceivably viral.

Of the 85 dogs, 72 had lesions in one or more organs or tissues, other than the liver and major reticuloendothelial organs. Sixty-one dogs had involvement of one or more of the major reticuloendothelial organs. Lesions were found in other than hepatic foci in dogs which had minimal levels of retained BSP in their serums and in those which retained a high percentage of dye.

DISCUSSION

All previous work with the BSP function test, using photoelectric methods, was based on 30-minute blood sampling and, therefore, direct comparison between the current results and those reported previously is not possible.

If the findings obtained from several of the dogs given drugs A and B are eliminated, tentative ranges of retention emerge and allow speculative normal and abnormal limits for interpretation. Under conditions of this experiment, the upper limit of retention in apparently normal beagle dogs, without hepatopathy, was 1.5 per cent; this was considered to be the upper normal limit. Retentions between 1.5 and 4.0 per cent should be considered suspicious or indicative of at least functional, if not mild cytologic, alteration in the liver. Retentions above 4.0 per cent are indicative of definite hepatic dysfunction in which cytologic evidence can be expected.

The fallibility of the function test in the lower retention bracket, *i.e.*, below 5.0 per cent, is seen (table 3). Of 52 dogs retaining 1.5 per cent or less BSP, 15 had very mild to moderate microscopic hepatic lesions. Three of 9 dogs having retentions of 1.8 to 2.5 per cent had mild lesions. Of the 5 animals with serum dye values between 3.6 and 4.3 per cent, 4 had mild to moderate cytologic changes in the liver. In the total evaluation, 68 dogs in this study had BSP retentions of less than 5.0 per cent and of these, 23 sustained very mild to moderate hepatic alterations. The remaining 17 dogs had retentions over 5.0

TABLE 3—Summary of Bromsulfophthalein Retention in Relation to Hepatic Lesions

% BSP retention	No. of dogs	Lesions	No. lesions
0.8	45	10	35
1.2	1	1	0
1.5	6	4	2
1.8	2	0	2
2.2	6	3	3
2.5	1	0	1
3.6	1	0	1
4.0	1	1	0
4.3	3	3	0
4.8	2	1	1
5.9	2	2	0
6.2	2	2	0
7.0	1	1	0
9.0	1	1	0
9.8	2	2	0
10.5	1	1	0
12.0	1	1	0
16.2	1	1	0
21.8	2	2	0
23.6	1	1	0
29.8	1	1	0
34.0	1	1	0
41.2	1	1	0

per cent, and all had moderately to markedly affected livers. Thus, the correlation between BSP retention and hepatic defects was inconsistent.

In carbon tetrachloride-intoxicated dogs, in which uniform correlation could be expected, there were inconsistencies between dye retention and intensity of the morbid hepatic state. One dog, although sustaining a moderate degree of hepatic damage, had dye retention in the normal range. The high retentions in the 4 other dogs was a more accurate indication of the acute cytologic alteration rather than a measure of the developing chronic reaction, because the dog with the most advanced cirrhotic state had the lowest retention.

In dogs given drug A, there was an inverse relationship between retention and the cytologic condition of the liver. Retention was uniformly slightly elevated and lesions were evident in the livers of only 2 of the 6 dogs. Retention of 1.5 per cent in 1 dog was not inconsistent with the mild focal subacute hepatitis, but the moderate perivasculitis in the other hardly justified the 9.0 per cent dye retention recorded. Cytologic evidence did not correlate with the slight retentions in the 4 remaining dogs. It is conceivable that this drug, which is an antibiotic, functionally altered the excretory activity of the liver without reflecting visible cellular change. This would further verify the conclusion of previous investigators as to the sensitivity of this test in detecting even subcytologic differences in hepatic function.

Five of the 8 dogs given drug B were free of microscopic hepatic lesions, and the degree of retention in all the animals varied with the dosage level. Dye retentions were not excessive. However, in 2 dogs with similar hepatic lesions, there was rather wide variation in the dye retained; one value was within the normal limits and the other was compatible with the injury.

In the dogs given drug C, there was good correlation of the two evaluation factors, with one exception. Retentions were within normal range and the livers were not abnormal, except in 1 dog in which the retention of dye was 41.2 per cent, the highest recorded for any dog in the study. The hepatic lesions would not appear to justify the high retention, and it was more likely the result of generalized circulatory collapse.

There was a more reciprocal agreement between retention and liver injury in the dogs given drugs D and E. Definite excretory failure was evident in the livers of dogs given drug D. There was no evidence of hepatopathy in the dogs given drug E and their test values were within the normal range, with the exception of 1 dog which had a mild focal hepatitis but an 0.8 per cent dye retention.

In the distemper-complex dogs, correlation of test and hepatopathy was poorest at the borderline of retention. Mild to moderate lesions without elevated BSP values were common in these dogs. Likewise, the type and, to a degree, the extent of hepatic lesions were not reflected in the clearance of dye from the blood stream in several of the dogs. One had focal hepatitis with necrotic foci yet had 0.8 per cent retention; whereas, another with similar defects had 4.0 per cent retention. In others, however, there was reasonably good correlation between retention and either the intensity or extent of the liver damage.

Only 1 dog inoculated with infectious canine hepatitis virus, and having notable hepatic defects, had a function test value within the normal range; in the others, lesions correlated with retention.

Fever, *per se,* in the dogs tested did not cause elevated retention. These findings are not in agreement with those of Noyan.[21]

There was no definite evidence that lesions in other organs, especially the primarily reticuloendothelial tissues, caused increased retention of BSP. A number of dogs had infrequent to frequent involvement of other organs and tissues with no hepatopathy and with normal retention. However, in those dogs with marked liver lesions, high retention, and nonhepatic lesions, this fact is less evident; there was no irrefutable evidence that at least part of the retention was not caused by lesions in the nonhepatic tissues.

When all facets of this study are considered, these facts pertaining to the function test emerge: (1) Correlation of dye retention and hepatic lesions was not consistent when either one was mild. (2) Dye retentions below 5.0 per cent were least correlative with the state of the liver; elevated BSP levels without detectable parenchymatous alterations were most commonly encountered (false positive), but the reverse also occurred (false negative). This was particularly true in those cases where evaluation was made in dogs given drugs or

afflicted with infectious diseases. (3) The test did not reflect the circumscribed granulomas of parasitic origin or allergic (eosinophilic) reaction in the liver. (4) Correlation between more severely affected livers and reduced blood clearance of the dye was only partially reciprocal and was not uniformly quantitative, as suggested by Gornall and Bardawell [11] and Hoerlein and Green.[13] (5) The results in this study essentially confirm Noyan's [21] conclusions that the BSP function test is most reliable and valuable in acute hepatopathies. (6) The test would appear to be of little value in the differential diagnosis of canine distemper and infectious hepatitis, because secondary toxic hepatitis is commonly encountered in the former disease. (7) In drug toxicopathology, the test cannot stand alone as an indicator of liver injury, even though retention of dye suggests alteration of hepatic function.

This evaluation of the BSP function test in the dog re-emphasizes the common fallibility of a single biological test in appraisal of a complex pathologic state.

SUMMARY AND CONCLUSIONS

1) The bromsulfophthalein (BSP) liver function test, following the procedure of Hepler, was performed in 85 dogs; the state of the liver was appraised by necropsy and histopathologic examination.

2) Dye retention was determined and pathologic evaluations were made in normal dogs, dogs given carbon tetrachloride, dogs given other drugs, dogs given infectious canine hepatitis virus, dogs with clinical evidence of distemper complex, and dogs with fever but without other readily detectable signs of disease.

3) Under conditions of the study, the normal limit of BSP retention was 1.5 per cent or under; retentions between 1.5 and 4.0 per cent were considered suspicious and those above 4.0 per cent were considered frankly abnormal.

4) Reciprocal agreement between retained BSP and cytologic hepatic alteration was inconsistent, especially at the lower range of retention. Twenty-nine per cent of the dogs with 1.5 per cent retention or less had detectable liver defects of varying degree. The two factors were generally, but not uniformly, correlative in the higher or abnormal range.

5) The test was most sensitive and correlative in acute hepatopathy.

6) When the test is used in toxicopathologic evaluation of drugs, the results should be interpreted with caution.

7) Excessively high dye retention, without correlative liver damage, occurred in cardiovascular collapse.

8) Pathologic states in other organs and tissues, including reticuloendothelial tissues, appeared inconsequential in relation to BSP retention.

9) Pyrexia, *per se*, was not a factor in reduced clearance of dye from the blood stream.

References

[1] Bauer, R. W., and Pessotti, R. L.: The Removal of Bromsulfalein from Blood Plasma by the Liver of the Rat. J. Pharmacol. & Exptl. Therap., 97, (1949): 358–370.

[2] Bauer, R. W., and Pessotti, R. L.: BSP Uptake and Excretion in the Dog. Am. J. Physiol., 162, (1949): 565–574.

[3] Bauer, R. W., Krebs, J. S., and Pessotti, R. L.: Bromsulfonphthalein as a Tool for Study of Liver Physiology. Fed. Proc., 9, (1950): 259.

[4] Cantarow, A., and Wirtz, C. W.: Excretion of Bromsulfalein in the Bile. Proc. Soc. Exptl. Biol. & Med., 47, (1941): 252–254.

[5] Casals, J., and Olitsky, P. K.: Tests for Hepatic Dysfunction in Mice. Proc. Soc. Exptl. Biol. & Med., 63, (1946): 383–390.

[6] Coffin, D. L.: Manual of Veterinary Clinical Pathology. 3rd ed. Comstock Publishing Associates, Ithaca, N.Y., 1953.

[7] Cohn, C., Levine, R., and Streicher, D.: The Rate of Removal of Intravenously Injected Bromsulfalein by the Liver and Extrahepatic Tissues of the Dog. Am. J. Physiol., 150, (1947): 299–303.

[8] Drill, V. A., and Ivy, A. C.: Comparative Value of Bromsulfalein, Serum Phosphatase, Prothrombin Time and Intravenous Galactose Tolerance Tests in Detecting Hepatic Damage Produced by Carbon Tetrachloride. J. Clin. Invest., 23, (1944): 209–216.

[9] Gaebler, C. H.: Determination of Bromsulfalein in Normal, Turbid, Hemolyzed or Icteric Serums. Am. J. Clin. Path., 15, (1945): 452–455.

[10] Giges, B., Mann, J. D., and Sharon, W. S.: Bromsulfalein Retention in Obstructive Jaundice. Proc. Soc. Exptl. Biol. & Med., 79, (1952): 375–376.

[11] Gornall, A. G., and Bardawell, C. J.: The Study of Liver Function in Dogs. Canad. J. M. Sci., 30, (1952): 256–271.

[12] Hepler, O. E.: Manual of Clinical Laboratory Methods. 4th ed. Charles C Thomas, Springfield, Ill., 1952.

[13] Hoerlein, B. F., and Greene, J. E.: The Bromsulfalein Liver Function Test as an Aid in the Diagnosis of Canine Hepatosis. North Am. Vet., 31, (1950): 662–665.

[14] Klein, R. I., and Levinson, S. A.: Removal of Bromsulphalein from the Blood Stream by the Reticulo-Endothelial System. Proc. Soc. Exptl. Biol. & Med., 31, (1933): 179–181.

[15] Lamson, P. D., and Wing, R.: Early Cirrhosis of the Liver Produced in Dogs by Carbon Tetrachloride. J. Pharmacol. & Exptl. Therap., 29, (1926): 191–202.

[16] Lamson, P. D., and Wing, R.: Blood Fibrin and Levulose Tolerance in Acute and Chronic Carbon Tetrachloride Intoxication. J. Pharmacol. & Exptl. Therap., 28, (1926): 399–408.

[17] Linder, G. C., Parker, R. G. F., and Selzer, G.: Bromsulfalein Retention in Young Rats on Diets Producing Liver Necrosis. Brit. J. Exptl. Path., 34, (1953): 656–660.

XUM

[18] McKibbin, J. M., Thayer, S., and Stare, F. J.: Choline Deficiency Studies in Dogs. J. Lab. & Clin. Med., 29, (1944): 1109–1122.

[19] Mills, M. A., and Dragstedt, C. A.: Bromsulphalein Dye Retention Test as a Measure of Functional Activity of Reticulo-Endothelial System. Proc. Soc. Exptl. Biol. & Med., 34, (1936): 228–231.

[20] Mills, M. A., and Dragstedt, C. A.: Removal of Intravenously Injected Bromsulfalein from the Blood Stream of the Dog. Arch. Int. Med., 62, (1938): 216–221.

[21] Noyan, Fethi: Observations on Some of the Liver Function Tests in Dogs. Ph.D. thesis, Ohio State University, Columbus, 1949.

[22] Pratt, E. B., Burdick, F. D., and Holmes, J. H.: Measurement of Liver Blood Flow in Unanesthetized Dog Using the Bromsulfalein Dye Method. Am. J. Physiol., 171, (1952): 471–478.

[23] Rosenthal, S. M., and White, E. C.: Clinical Application of Bromsulphalein Test for Hepatic Function. J. Am. M. A., 84, (1925): 1112–1114.

[24] Svirbely, J. L., Monaco, A. R., and Alford, W. C.: The Comparative Efficiency of Various Liver Function Tests in Detecting Hepatic Damage Produced in Dogs by Xylidine. J. Lab. & Clin. Med., 31, (1946): 1133–1143.

[25] Wirtz, C. W., Cantarow, A., Snape, W. J., and Delserone, B.: Bile Volume and Excretion of Pigment and Bromsulfalein in Dogs Receiving Carbon Tetrachloride. Am. J. Physiol., 165, (1951): 680–687.

SUMMARIO IN INTERLINGUA

Evalutation del Test de Function Hepatic a Sulfobromophthaleina in Canes

1. Le test de function hepatic a sulfobromophthaleina esseva executate in 85 canes secundo le methodologia de Hepler. Le stato del hepate esseva estimate in examines necroptic e histopathologic.

2. Le retention del colorante esseva determinate e le evalutation pathologic esseva interprendite in canes normal, in canes tractate con tetrachloruro de carbon, in canes tractate con altere drogas, in canes al quales virus de infectiose hepatitis canin habeva essite administrate, in canes con indicationes clinic del complexo de maladia canin, e in canes con febre sed sin altere prestemente recognoscibile signos de morbo.

3. Sub le conditiones del studio, le limite normal del retention de sulfobromophthaleina esseva 1,5 pro cento o minus. Retentiones inter 1,5 e 4,0 pro cento esseva considerate como suspecte, e valores de plus que 4,0 pro cento indicava un stato francamente anormal.

4. Le congruentia del retention de sulfobromophthaleina con le alterationes cytologic del hepate e vice versa esseva pauco uniforme, particularmente in le ordines pauco elevate del retention. Vinti-novem pro cento del canes con 1,5 pro cento de retention o minus habeva detegibile defectos hepatic de varie grados. Le duo factores esseva generalmente (sed non uniformemente) correlationate in le ordines plus elevate e anormal.

5. Le test esseva le plus sensibile in acute hepatopathia. In iste casos, etiam le correlation esseva melior.

6. Quando le test es usate in le evalutation toxicopathologic de drogas, le resultatos debe esser interpretate cautissimemente.

7. Excessivemente alte retentiones del colorante occurreva sin correlative lesiones hepatic in collapso cardiovascular.

8. Statos pathologic in altere organos e tissus, incluse le tissus reticuloendothelial, pareva esser sin interesse in relation al retention de sulfobromophthaleina.

9. Pyrexia per se non esseva un factor reduceente le clearance del colorante ab le circulation del sanguine.

Enteroviruses of Swine.
II. Studies on the Natural History of Infection and Immunity

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THE RECOVERY of a specific kind of enterovirus of swine, apparently heretofore unrecognized, was published in a previous report.[1] Of 376 strains of enterovirus recovered from the feces of apparently healthy young pigs, all except one belonged to the same serotype. Other observations, also reported in the previous paper, indicated that infection with the virus occurred very early in the lives of pigs raised at the Municipal Farm. Colostrum obtained from brood sows contained specific antibodies against the enterovirus. Pigs suckled by these sows experienced inapparent infections and excreted virus in the feces for long periods of time.

Some additional observations have been made on the natural history of infection for swine enterovirus. The large number of pigs born and raised at the Municipal Farm provided a stable population suitable for studies of herd infection and immunity. A number of studies were made in order to determine (*a*) if any illness or pathologic lesions observed in pigs that died at the farm were associated with the presence of enterovirus in tissues, (*b*) the patterns of virus excretion and of antibody development in young pigs and (*c*) if common houseflies (*Musca domestica*) obtained at the farm were carriers of enterovirus.

MATERIALS AND METHODS

A description of the farm and a résumé of practices applied to the breeding and raising of pigs appeared in the preceding report.[1] The field studies described in this report were carried out at the Municipal Farm at Kansas City, Missouri, except for a serologic survey in which serums were collected from pigs in herds located elsewhere in Missouri.*

Prototypic Virus and Antiserums.—One of the earliest isolates obtained from the feces of a young pig was selected as a prototypic virus. This strain, designated MF (Municipal Farm), was used to form a large stock of virus for use in subsequent tests. The history of passage and the manner in which this stock was formed have been described.[1] An antiserum was also prepared in rabbits with the MF strain of virus. This antiserum, used to type all viruses obtained from pigs, had a titer of 1:256, using approximately 100 t.c.d.$_{50}$ (50% tissue culture dose) of the stock virus in neutralization tests.[6]

Serums.—Blood was obtained by venapuncture, using the anterior vena cava in young pigs and the posterior auricular vein in older animals. The serum fraction was removed from the clot and stored at −20 C.

Colostrum.—Approximately 10 ml. of colostrum was collected from sows nearly ready to farrow, or within one day of farrowing. Samples of colostrum were stored at −20 C.

Feces.—Fecal samples were obtained on rectal swabs. Swabs were placed in test tubes containing 2 ml. of maintenance medium (MM)[1] and stored at −20 C. until the day of test. After thawing, swabs were twirled in this fluid and the residual fluid was expressed by rolling swabs against the glass. The turbid fluid was centrifuged (2,500 r.p.m./30 min.); the supernatant fluids were decanted for use in tests.** Feces obtained from the lumen of the colon at necropsy were homogenized to make a 10 per cent suspension (wet weight); thereafter, these samples were treated in the same way as samples obtained on rectal swabs.

Nasal Swabs.—The nasal swabs were processed and prepared using the methods described for rectal swabs.

Tissues.—Each tissue (or body fluid) obtained at necropsy was removed with separate sterile sets of instruments, using care to avoid contamination of tissues by contents of the intestine. Some-

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Data from these studies were submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Kansas (Beran).

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** The concentrations of antibiotics added to each milliliter of fecal and tissue suspensions were: penicillin, 200 U.; streptomycin, 50 mg.; and butyl parasept, 6 mg.

times it was not possible to avoid contamination of tissues with other adventitious fluids; e.g., peritoneal exudate. After removal of segments of the alimentary tract, the contents of each were removed by gently stripping the segment manually. Segments of the alimentary tract and their contents were handled separately. Each tissue was washed with a volume of MM about 20 times greater than the mass of tissue. The tissues were then ground in a mortar with a pestle using alundum as abrasive, and suspended in MM to make a 10 per cent extract. The contents of intestinal segments, the whole blood samples, and the exudates were diluted with 9 parts of MM. The several extracts were spun (2,500 r.p.m./30 min.) in a centrifuge and the supernatant fluids were obtained for study. All samples were placed in sterile, rubber-stoppered vials and stored at −20 C.

Flies.—Flies were obtained at the Municipal Farm during early morning hours by lightly swatting them as they rested on posts or partitions and collecting them after they fell on sheets of paper. Nine samples of flies were collected from three locations on three different days and stored separately at −20 C. About 75 per cent were alive at collection; crushed flies were discarded. Flies were prepared for tests in two ways. First, 25 flies obtained from each of the nine samples were ground to a paste in a mortar, and prepared in 10 per cent suspension with phosphate buffered saline solution (PBS).[5] Second, in order to measure the concentrations of virus on or within component parts of flies, two separate groups of flies (each consisting of 10 flies from eight samples and 20 flies from one sample) were dissected, using a dissecting microscope. The heads, thoraces, wings, abdomens, and legs from flies of each group were collected in separate pools; tissues in each pool were washed four times with PBS in a volume 10 times the weight of the sampled parts. The tissues were separated from the wash fluid by light centrifugation and ground in a sterile mortar with a pestle. The homogenized whole flies or dissected flies were centrifuged at 14,800 r.p.m. for 30 minutes in a refrigerated centrifuge; the supernatant fluids were tested for virus.

Measurements of Virus and Antibody.—All measurements were made in cells derived from fetal pig kidneys,[1] prepared by the method of Youngner.[9] In screening for the presence or absence of virus, each sample was inoculated into three cultures (0.15 ml./culture). For determining the presence and the amounts of virus, all samples were tested initially in a 1:10 dilution and thereafter were titrated using serial tenfold dilutions; 0.1 ml. of each of these dilutions was delivered into cultures, using three cultures per dilution. End points were determined on the basis of cytolytic scores obtained on the fifth or sixth day after inoculation.

For antibody measurements, serums were heated at 56 C. for 30 minutes in order to inactivate virus or nonspecific inhibitors. All serums were tested first using a single dilution; serums collected from living pigs were diluted 1:4 and serums from dead pigs were diluted 1:8. The volume of serum usually obtained from dead suckling pigs was small, *i.e.*, 0.5 to 1.0 ml. When serum dilution end points (s.d.e.) were determined, serums were diluted in fourfold steps. Approximately 200 $t.c.d._{50}$ of the MF strain was added to each diluted serum; the final concentration of virus was about 100 $t.c.d._{50}$/0.1 ml., which was the volume delivered into cultures. Three cultures were used per dilution. The 50 per cent end points for virus infectivity ($t.c.d._{50}$) and s.d.e. were determined by the method of Reed and Muench.[7]

RESULTS

Clinical Records of Illness and Isolation of Virus.—During the 12-month period of observation, the death rate among pigs from birth to weaning (3 weeks of age) averaged only 10.8 per cent. The only occurrence of clinical illness observed during the study period was a mild epizootic of enteritis which occurred during June, 1957. It involved more than 50 per cent of pigs less than 2 months of age and was characterized by fluid yellow diarrhea associated with moderate dehydration. Clinical signs persisted for one month in about 20 per cent of the affected pigs.

On June 13, 1957, during the period when enteritis was prevalent among pigs, fecal samples were obtained from 28 pigs, 1 to 2 weeks of age, and from 13 pigs 5 to 6 weeks of age. Enterovirus was not recovered from any of the younger pigs; 12 of these pigs had "scours"; 16 were apparently healthy. Among the 13 pigs 5 to 6 weeks of age, 4 yielded enterovirus; 3 of 11 pigs with diarrhea and 1 of 2 apparently healthy pigs yielded enterovirus also. The results of these studies indicated that the occurrence of enteritis was not correlated with the presence of enterovirus in feces. The frequency of recovery of enterovirus from the feces of pigs during the outbreak of enteritis did not differ significantly from recovery rates during periods when enteritis was absent in the herd.

Atrophic rhinitis (AR), a disease of unknown cause, was enzootic in the herd; an increased clinical incidence occurred during the epizootic of enteritis. This disease had another peak, unassociated with enteritis, in 5 to 6-week-old pigs during December, 1956, and January, 1957. The incidence of clinical AR did not have any correlation with the incidence of enterovirus in the feces of pigs. Studies of 70 nasal swabs, about one half of which were obtained from

young pigs having clinical signs of AR, failed to yield enterovirus.

The data summarizing the mortality rates, frequency of isolation of enterovirus, and kinds of clinical illness in young pigs from birth to 6 weeks of age are given (table 1).

Infection Rates in Sows and Their Litters.—Fecal samples obtained from 37 sows at the same time that their litters were sampled (within two weeks after farrowing) yielded six isolates of enterovirus. Of the 44 pigs farrowed by the 6 sows excreting enterovirus, 8 (18 per cent) had virus in fecal samples; of 214 pigs farrowed by 31 sows in which enterovirus was not detected in feces, 13 (6 %) were also found to have virus in feces.

In order to determine if enterovirus was present in the alimentary tracts of pigs prior to birth, samples of meconium were obtained from 33 pigs immediately after birth during the months of May, July, and August. During July, samples of meconium were also obtained from 6 fetuses aborted from 2 sows during the last month of pregnancy; enterovirus was not detected in fetal meconium.

Relationship of Enterovirus to Mortality in Pigs.—At the Municipal Farm, 31 pigs which died when less than 19 days old were studied at necropsy. The chief abnormalities in 23 pigs were encountered in lymph nodes and consisted of hemorrhagic lymphadenitis, chiefly involving mesenteric (20 pigs) and, less frequently, cervical, axillary, and inguinal nodes (14 pigs). In 3 pigs, only peripheral lymph nodes were involved; in 9, only mesenteric lymph nodes were involved; and in 11, both groups of lymph nodes were involved. Fifteen of the pigs had subcapsular and parenchymal hemorrhages in mesenteric lymph nodes; in 5 of the pigs only subcapsular ecchymoses were found. In these same 15 pigs, the colonic lymph nodes lying in the mesentery between the coils of the spiral colon were black (pseudomelanosis). Other abnormalities, less frequently encountered and not always associated with lymphadenitis, were virus pneumonia of pigs (VPP),[3] caseous pneumonia, hyperemia of turbinates (probably indicative of early pleuritis), ecchymosis in the mucosa of the alimentary tract, peritonitis, and atrophic rhinitis. No visible abnormalities were observed in 3 pigs. Starvation may have been the cause of death, either primarily or secondarily, in 2 of the 3 pigs in which abnormalities were not found and in 7 pigs observed to have lymphadenitis.

Only 1 of the 31 pigs yielded enterovirus; virus was detected in pooled samples of jejunum and spiral colon. The mucosa of the alimentary tract of this pig appeared normal. This pig died at 12 days of age,

TABLE 1—Summary of One Year's Observations of Pigs Born at the Municipal Farm; Number of Live Births, Incidence of Enteritis, and Rhinitis and Frequency of Detection of Enterovirus in Feces

		Age of pigs during period of observation										
		From birth to 4 weeks						5 to 6 weeks*				
					Virus isolations					Virus isolations		
						Positive tests					Positive tests	
Month	No. of live births	Enteritis (scours) (%)	Atrophic rhinitis (%)	Deaths (% of live births)	No. tested	No.	%	Enteritis (scours) (%)	Atrophic rhinitis (%)	No. tested	No.	%
Nov., 1956	289	....	..	15	28	0	....	....	....	....	....	....
Dec., 1956	448	....	..	16	32	0	....	10	10	23	9	39
Jan., 1957	402	....	..	15	30	3	10	...	5	27	19	70
Feb., 1957	456	....	..	11	25	0	....	....	<5**	27	11	41
March, 1957	475	....	..	4	28	0	....	....	<5	27	10	37
April, 1957	228	....	..	18	26	5	19	....	<5	24	20	83
May, 1957	816	....	..	5	19	3	16	....	<5	24	12	50
June, 1957	769	50	5	5	28	0	....	75	5	13	4	31
July, 1957	351	....	..	19	27	4	15	20	10	26	9	35
Aug., 1957	436	....	..	22	29	4	14	....	<5	21	5	24
Sept., 1957	283	....	..	24	25	0	....	....	<5	22	14	64
Oct., 1957	565	...	..	19	28	3	11	...	<5	22	16	73
Nov., 1957	.....	....	..	....	....	..	....	....	<5	26	25	96

The study was designed for a one-year period of observation. The open panel in the bottom row indicates that the same pigs born in October were observed at 5–6 weeks of age in November; the open panel in the top row has a similar explanation for pigs born in November of the preceding year.

* Deaths were not recorded among pigs in this age group. ** <5 for atrophic rhinitis indicates the sporadic occurrence of atrophic rhinitis in varying clinical severity, making it possible only to estimate the incidence.

probably from a pyogenic infection ("navel ill"), since pus and bacteria were obtained from distended joint spaces. A cytopathogenic agent was not detected in the pus obtained from the joints. Enterovirus was not detected in mucosae of the alimentary tracts obtained from the other 30, or in other tissues or body fluids obtained from any of the 31 pigs (table 2).

Twenty-two serums obtained from these 31 pigs were tested for the presence of antibodies against the MF strain enterovirus. Neutralizing antibodies were present in the serums of 16 pigs that died before 12 days of age (mean age, 3.7 days). Of the positive serums, 15 were obtained from pigs for which the cause of death was not determined; one came from a pig which may have died of starvation. The serum of the pig which harbored enterovirus in the intestines did not have antibodies against the MF strain; neither did four other serums from pigs which may have starved, nor were antibodies found in a single serum from a pig with caseous pneumonia.

The presence of antibodies against the MF strain in the serums of these 16 pigs without accompanying presence of enterovirus infection in the alimentary tract strongly suggested that these seropositive pigs obtained their antibodies in colostrum from immune dams. The results of the studies on these 31 pigs necropsied at the Municipal Farm are summarized (table 2).

Distribution of Enterovirus in Tissues of Pigs.—Thirteen pigs were killed in order to search for any visceral abnormalities which might be associated with the presence of enterovirus in the terminal colon and to delineate tissues in which enterovirus might be found during this early period of life. Enteroviruses neutralized by MF antiserums were recovered from the contents of the terminal colon in 6 of these 13 pigs; 3 pigs were 3 to 6 weeks of age, and 3 were 4 months of age (table 3).

In addition to the presence of virus in the contents of the terminal colon, enterovirus was found in the washed wall of this segment of the alimentary tract in all 6 pigs. Virus was also detected in the walls of the spiral colons of the 3 younger and of 1 of the 3 older pigs. Virus was recovered from the jejunum (2 pigs) and ileum (1 pig) of

TABLE 2—Kinds of Abnormalities and Their Distribution in 31 Pigs Dying of Various Causes at the Municipal Farm

		Age at death (days)						
Tissues	Kind of abnormality	Birth to 2 days	3 to 5	6 to 9	10 to 15	16 to 19	Totals	Virus isolations*
Turbinates	hyperemia	1/8**	1/11	1/4	1/5	0/3	4/31	0/4
Lungs	pneumonia (VPP)	1/8	7/11	4/4	3/5	1/3	16/31	ND***
	pneumonia (caseous)	0/8	0/11	0/4	2/5	3/3	5/31	0/2
	pleural exudate†	0/8	1/11	0/4	3/5	2/3	6/31	0/2
Stomach	intact mucosa, no milk‡	3/8	3/11	1/4	2/5	1/3	10/31	ND
	mucosal hemorrhage	1/8	1/11	0/4	0/5	0/3	2/31	0/2
Intestines	mucosal hemorrhage	0/8	1/11	1/4	0/5	0/3	2/31	1/31§
	peritoneal exudate†	0/8	1/11	0/4	1/5	0/3	2/31	0/2
Lymph nodes	mesenteric adenitis	5/8	7/11	3/4	3/5	2/3	20/31	0/12
	regional adenitis #	3/8	7/11	2/4	2/5	0/3	14/31	
Kidneys	subcapsular petechiae	2/8	3/11	1/4	0/5	0/3	6/31	0/5
Adrenals	enlarged	2/8	1/11	2/4	0/5	0/3	5/31	0/3
Joints	arthritis, purulent	0/8	0/11	0/4	1/5	0/3	1/31	0/1
Blood	.....	.....	.....	.....	.....	.....	.....	0/2
Presence of neutralizing antibodies		5/7	8/9	3/4	0/2	ND	16/22	.....

* Numerator = No. positive; denominator = No. tested. All serums were tested at the 1:8 dilution against 100 $t.c.d._{50}$ of the MF strain of swine enterovirus. The volume obtainable from 9 pigs after death at varying ages was insufficient for use in serum neutralization tests.

** Numerator = No. of pigs with lesions; denominator = No. of pigs necropsied. The underlined figures indicate that samples for serum neutralization tests (last column) were obtained from pigs.

*** ND = not done. Tests for virus in lungs from 8 other pigs with pneumonia (presumably VPP) were negative.

† Fibrinous adhesions were present.

‡ Indicates that pigs were unable to suckle for an indefinite period prior to death.

§ Samples of washed wall of jejunum and spiral colon were pooled for test in tissue culture. Except for the intestines, only tissues obtained from pigs with lesions were tested for cytopathogenic agents.

Cervical, axillary, and inguinal lymph nodes.

TABLE 3—Frequency of Detection of Enterovirus in Various Tissues Obtained from 6 Pigs Known to be Intestinal Carriers of Virus

Tissue	3 to 6 weeks old (3 pigs)		4 months old (3 pigs)	
	No. positive	Titer*	No. positive	Titer*
Blood	0	....	0	....
Lung	0	....	0	....
Heart	0	....	0	....
Liver	0	....	0	....
Spleen	0	....	0	....
Kidney	0	....	0	....
Urinary bladder	0	....	0	....
Mesenteric lymph nodes	0	....	0	....
Brain	0	....	ND	....
Stomach	0	....	0	....
Duodenum	ND†	....	0	....
Duodenal contents	ND	....	0	....
Jejunum	2	2.2	0	....
Jejunal contents	1	1.5	0	....
Ileum	1**	1.5	0	....
Ileac contents	0**	....	0	....
Spiral colon	3	4.0	1	3.5
Spiral colon contents	3	4.7	1	2.5
Terminal colon	3	3.2	3	2.3
Terminal colon contents	3	3.2	3	3.2
Serum neutralizing antibodies	3	1:48	3	1:270

* Virus titers expressed as $\log_{10}$ t.c.d_{50} per 0.1 Gm. tissue; serum titers expressed as 50 per cent serum dilution end point for 0.1 ml. serum against 100 t.c.d_{50} of virus, expressed as the averages of determinations for each of the 3 pigs in each age group.

** The ileum and ileac contents of only 1 of the 3 pigs in this age group were tested.

† ND = not done.

pigs 3 to 6 weeks of age, but was not recovered in the upper alimentary tract of 4-month-old pigs.

The frequency of detection of enterovirus, and the concentrations of virus found in *(a)* several segments of the alimentary tract and in *(b)* eight other visceral organs and blood are summarized (table 3). Several interesting points appear in the data. First, the constancy of finding virus in tissues of the alimentary tract of infected pigs and the uniform failure to detect virus in organs outside the alimentary tract suggested local infection and multiplication of virus in the intestinal tract. Since we are unable to give the time of onset of enterovirus infection in these pigs, we cannot be certain that organs outside the alimentary tract may not have been invaded by the virus during the initial stages of infection. A second point of interest is that enterovirus was recovered more uniformly from tissues of the small intestines of the younger pigs than in those of older pigs. Third, enterovirus was more uniformly recovered from tissues of the spiral colon of the younger pigs and in concentrations higher than were found in older pigs. The concentration of virus was highest in the spiral colons of the younger pigs and in the terminal colons of the older pigs. Fourth, all 6 of these pigs had neutralizing antibodies in their serums, ranging in titer from 1:16 to 1:512. The highest titers were found in the older animals. A specific association was not found between the level of antibody and the concentration of virus in the intestinal tracts of individual pigs.

The 3 pigs (3 to 6 weeks of age) from which enterovirus was recovered were emaciated, dehydrated, and had roughened haircoats. One had clinical manifestations of rickets. At necropsy, the cervical and mesenteric lymph nodes from 1 pig contained subcapsular hemorrhages. The 3 pigs (4 months of age) also excreted virus but were apparently healthy. At necropsy, no visible abnormalities were seen. The alimentary tracts of these 6 pigs were normal in appearence.

The other 7 pigs (3 to 6 weeks of age), from which enterovirus was not recovered, were also stunted and emaciated. Four had unusual clinical features; their heads deviated to one side and they circled and "bicycled" in lateral recumbency. Enterovirus was not recovered from the brains of these 4 pigs. In 1 of the 7 pigs, fibrosis and induration of the entire small intestine were seen; enterovirus was not found in the affected tissues. None of the 7 pigs had any other visible abnormalities in the alimentary tract or in any other organ.

The presence or absence of enterovirus in the alimentary tracts of these 13 pigs was not associated with any specific lesions in the organs examined.

The Distribution of Antibodies According to Age.—Colostrum was obtained from 8 gilts just prior to, or immediately after, farrowing. Neutralizing antibodies were found in the colostrum of all 8 gilts; the mean s.d.e. was 1:90 and the range was 1:4 to 1:256. The 33 pigs borne by these gilts and removed from their mothers immediately after birth did not possess in their serums antibodies neutralizing the MF strain.

The 33 newborn pigs were used in other studies [2] and were not studied for changes in natural antibody status. In order to define the distribution of antibodies in pigs maintained at the Municipal Farm, serums were obtained from 60 different pigs, 1 day to 1 year old. Pigs acquired neutralizing

TABLE 4—Distribution of Neutralizing Antibodies in Colostrum and in Serums Obtained from Pigs of Various Ages at the Municipal Farm

Age	Sample: Kind	Sample: No.	% of samples neutralizing MF strain	No. of samples with dilution end points* in ranges: <4	4–16	>16–64	>64–256	>256	Mean s.d.e.**
8–12 mos.	colostrum	8	100	....	3	2	3	..	90
<1 day†	serum	33	0	33	..	..	..	..	0
1–10 days‡	serum	11	82	2	3	4	2	..	43
3–6 wks.	serum	5	100	....	3	2	..	..	32
6–12 wks.	serum	8	100	....	3	3	2	..	66
3–6 mos.	serum	22	95	1	11	5	4	1	89
6–12 mos.	serum	14	100	....	7	6	1	..	47
Totals—serums of pigs receiving colostrum				3	27	20	9	1	63

* Reciprocal of final dilution neutralizing 100 t.c.d.$_{50}$ of enterovirus. ** s.d.e. = serum dilution end points. † Pigs sampled before they received colostrum. ‡ All pigs entered below this symbol were allowed to suckle for 21 days before weaning.

antibodies by at least 10 days of age*; antibody levels were encountered thereafter without any appreciable change in titer up to 1 year of age. Only 3 of the 60 pigs did not have antibodies in serums initially diluted at 1:4. While the s.d.e. differed greatly in individual pigs, the mean titers obtained for the different age groups were not significantly different. Antibody levels in colostrum and serum samples obtained according to age of pigs are shown (table 4).

Distribution of Antibodies in Other Herds.—Serum was obtained from blood collected from 186 pigs, 2 to 24 months old, at 41 farms distributed in 35 communities in Missouri. The sampling of each herd consisted of serums representing 3 to 5 pigs. These serums were diluted 1:4 and tested for neutralizing antibodies against the MF strain.

Serums obtained from 134 pigs unequivocally neutralized 100 t.c.d.$_{50}$ of the MF strain. Fifty-three per cent of pigs 2 to 5 months of age and 81 per cent of pigs 5 months to 2 years of age were seropositive. The seropositive herds were distributed widely in Missouri; distance-wise, the nearest was 20, and the furthest 165 miles from the Municipal Farm. The 52 (39%) seronegative pigs were 11 pigs in three herds in which no evidence of enterovirus infection was found, and 41 pigs dispersed among seropositive animals in 23 herds. The three seronegative herds were on farms located within 50 miles of the Municipal Farm.

Differences in sex incidence of neutralizing antibodies in pigs were not observed; 77 females (78%) and 43 males (72%) were seropositive. Although differences were observed in the presence or absence of antibodies among pigs of different breeds, the numbers entered for each breed were too small to show significant differences. The serologic studies on the pigs surveyed in several communities in Missouri are summarized (table 5).

TABLE 5—The Relative Frequency of Detecting Antibodies in 186 Pigs of Various Ages and Sex in Missouri

Age of pigs	Male: No. serums	Male: % positive*	Female: No. serums	Female: % positive	Both sexes: No. serums	Both sexes: % positive
2 to 5 mo.	8	50	11	54	19	53
5 to 6 mo.	22	68	42	86	64	81
6 to 12 mo.	6	83	17	82	23	83
1 to 2 yr.	7	100	7	57	14	79
unknown	....	....	....	....	66	64
Totals and average percentages	43	72	77	78	186	72

* Serums were tested at the 1:4 dilution against 100 t.c.d.$_{50}$ of MF virus. The positive tests were scored on the basis of 0/3 tubes showing cytopathic changes. The tests on individual pigs showing partial neutralization, *i.e.*, 1 or 2 of 3 tubes showing cytopathic changes, were scored as negative.

Recovery of Enterovirus from Flies.—About 750 common houseflies *(M. domestica)* were collected on three different days (October, 1957) at three different sites at the Municipal Farm. These were collected in two barns containing 3- to 7-week-old pigs and in an open barn containing 7- to 13-week-old pigs. The flies were pooled according to days and sites of collection.

Enterovirus was detected in homogenized pools of whole flies obtained at each of the three sites on the three different days. The mean concentration of virus per fly in the nine pools was 93 t.c.d.$_{50}$ per fly; the mean

* The exact time in the life of these pigs when antibody appeared in the serum is unknown. Young *et al.*,[8] in a study of passive transfer of antibodies from colostrum, found antibodies in blood of baby pigs during the first 6 to 8 hours of life.

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values for the individual pools ranged between 19 and 186 t.c.d.$_{50}$ per fly.

The concentrations of enterovirus on, or in, the legs, wings, heads, thoraces, and abdomens of flies are recorded (table 6).

TABLE 6—The Detection of Enterovirus on the Surface and Within Tisues of Houseflies (Musca domestica) Collected at the Municipal Farm

Inoculum**	Average concentration of virus recovered per fly*					
	Legs	Wings	Head	Thorax	Abdomen	Total
WASH FLUID						
Fly pool 1	0.5	....†	0.3	6.4	205.0	212.3
Fly pool 2	0.3	....	0.4	15.3	19.9	36.0
Mean	0.4	....	0.4	10.9	112.5	124.2
GROUND TISSUES						
Fly pool 1	0.1	....	....	.....	32.5	32.6
Fly pool 2	....	....	....	.....	99.9	99.9
Mean	0.1	....	....	.....	66.2	66.2
TOTAL RECOVERED						
Fly pool 1	0.7	....	0.3	6.4	237.5	244.9
Fly pool 2	0.3	....	0.4	15.3	119.8	135.8
Mean	0.5	....	0.4	10.9	178.6	190.4

* Expressed as arithmetic mean of numbers of t.c.d.$_{50}$ of virus recovered per fly, based on average values for 100 flies. ** Inoculum was 0.1 ml. of 10^{-1} suspension based on wet weight of flies. † Insufficient virus to cause cytopathic effect in tissue culture.

Large amounts of virus were removable in "wash" fluids (although most flies were intact at the time of collection, the methods used did not preclude the contamination of external body surfaces by oral or fecal excreta, or contamination from lacerated viscera during dissection). Virus was present in the first wash fluid obtained from all segments except the wings; virus was never detected on or in wings during these studies. The second wash fluid yielded virus only from the abdomens of flies of one pool. None of the fluids from the third and fourth washings of the five anatomical parts of flies contained detectable amounts of enterovirus. The largest concentration of enterovirus removed by washing the component parts of the body was obtained from the abdominal segments, yielding an average of 112 t.c.d.$_{50}$ per fly as compared to a mean total per fly of 11 t.c.d.$_{50}$ from the thoraces and less than 1 t.c.d.$_{50}$ from the legs and heads.

After homogenization of these five anatomical segments (each washed four times with PBS), enterovirus was found only in the abdominal segments. The mean concentrations of virus in the abdomen was 66.0 t.c.d.$_{50}$ per fly. In contrast to the large amounts recovered from the abdominal segments, detectable amounts of enterovirus were not recovered from thoraces, heads, or wings, and only barely detectable quantities of virus (0.1 t.c.d.$_{50}$ per fly) were recovered from legs of flies in one pool. The data on the recovery of enterovirus from whole flies and from their designated anatomical parts are given (table 6).

DISCUSSION

In a preceding report [1] we described an enterovirus of swine heretofore unidentified; this virus was found in feces obtained from many young pigs in a herd of swine. The prototypic strain (MF) was found to be unrelated to viruses known to cause disease of swine, and to some other viruses associated with diseases of other domestic animals. The MF strain of virus was unrelated to 38 serologically distinctive enteroviruses recovered from human beings.[4] The data reported in this paper were obtained in order to add to some previous observations [1] on infection and immunity arising from swine enterovirus.

Swine enterovirus was excreted from the alimentary tracts of young pigs, particularly during the first 2½ months of life. The over-all chance of detecting virus in suckling pigs was 6.8 per cent; by the time pigs were 5 to 6 and 9 to 10 weeks of age, the rates of recovering virus in feces increased to 55 and 73 per cent, respectively (table 1).[1] Based on recovery of virus from monthly samples obtained from the same pigs, an estimate can be obtained on the duration of infection of the alimentary tract. Thirteen pigs excreted virus during the first 2 weeks of life; 6 of these pigs were still excreting virus four weeks later, and 10 were excreting virus at 10 weeks of age. Among 115 additional pigs first found to be excreting virus at 5 to 6 weeks of age, 87 were still excreting virus one month later. For another group of 37 pigs sampled when they were 8 to 12 months of age, only 6 (16%) were excreting virus in feces.

These studies on the presence of enterovirus in feces of pigs indicated that the risk of acquiring alimentary infection was delayed in the first weeks of life and thereafter increased in the early months of life, when all or nearly all pigs were infected. For pigs 8 to 12 months of age, the incidence of virus excretion declined; only 16 per cent of pigs (about 1 year of age) were found to excrete virus. The duration of the carrier state lasted at least one month for

most pigs; however, information is not yet available on how much longer the carrier state might last in individual pigs. For most pigs, the duration was estimated to be two or three months and possibly for as long as 6 months. However, we do not know if recurrent infection of the alimentary tract occurred in older pigs, which might explain the presence of virus in feces of these animals.

Antibodies neutralizing the MF strain were almost uniformly present in suckling pigs, and the presence of such antibodies was correlated with the presence of antibodies in colostrum received from the dam. Colostrum or serums obtained from 22 sows contained specific antibodies (table 4). Twenty-five of the serum samples obtained from 33 young pigs (11 samples from living pigs listed in table 4 and 22 samples from dead pigs listed in table 2), 3 to 12 days of age, neutralized the MF strain. Probably not all of these pigs had obtained colostrum, since 5 of the 22 dead pigs (3 to 7 days old) did not have milk present in the alimentary tract. Since none of these 33 pigs yielded virus in feces, the serum antibodies must have been passively acquired in colostrum obtained from seropositive dams rather than from a specific primary antigenic stimulus. Serums obtained from 33 other pigs, removed from the sows prior to receiving colostrum, did not have specific serum neutralizing antibodies.

No appreciable changes in antibody status were found in pigs 1 week to 1 year old. During this period, the number of pigs having specific serum neutralizing antibodies remained relatively constant (table 4). The frequency of recovery of enterovirus during this period was nil at birth and significantly lower during the first few weeks of life than thereafter, suggesting that antibodies acquired from colostrum passively protected the younger pigs from alimentary infection. By 2 to 3 months of age, the majority of pigs experienced active infection of the alimentary tract, and very likely this antigenic stimulus resulted in active immunization. These temporal relationships between infection and rate of acquiring antibody suggested that active immunity closely followed or overlapped the loss of passive immunity in young pigs.

The course of infection in swine infected with enterovirus was not associated with any discrete clinical illness. Young animals acquired infection without showing visible abnormalities in any specific organ which could be consistently associated with the presence of enteroviruses in the alimentary tract.

Some of the natural events in the history of infection of pigs with swine enterovirus may be reconstructed, based on results applying largely to conditions prevailing at the Municipal Farm. The virus was widespread in pigs and in their habitat. The infection was independent of recognizable illness, and was principally associated with tissues of the lower alimentary tract of young pigs. The risk of acquiring infection during the first weeks of life, when pigs were suckling, was modified by antibodies acquired in colostrum obtained from the dam. The antibodies in colostrum, for a brief time, probably neutralized virus entering the alimentary tract. When these antibodies were no longer available to the suckling pig (or at weaning), multiplication of virus occurred within susceptible cells in the alimentary tract. While passively acquired antibodies afforded no appreciable protection to susceptible cells in the alimentary tract, they may have protected cells in other organs, thereby protecting pigs against illness and death. No evidence was obtained in the study of spread of virus to other tissue loci, although in another study [2] virus was detected in other organs without evidence of cellular injury. In this latter study,[2] we found that active immunity may develop in pigs as young as 14 days of age. Thus, an early onset of infection may lead to active immunization while many pigs are still partially protected by antibodies acquired in colostrum. There is a transitional period, not fully defined, wherein primary antibody response (induced by infection) occurs before all the passively acquired humoral antibodies (from colostrum) are lost.

Swine enterovirus was widely dispersed in the environment of the Municipal Farm, and in herds of swine located elsewhere in Missouri. The high infection rates were principally due to the continuous presence of large concentrations of virus in the natural environment and to the rapid dispersal of virus from infected to susceptible young pigs. The excretion of virus by young pigs for long periods, along with a smaller reservoir of active infection in older pigs, assured perpetuation of infection, at least in this population, into which pigs were born daily. Besides direct spread of virus from

pig to pig, infection may have been transported to pigs indirectly by common houseflies *(M. domestica)* or by farm laborers who went from pen to pen and barn to barn while wearing the same boots and clothes and using the same cleaning tools.

SUMMARY

Swine enterovirus (MF strain) is an infectious agent. In young pigs, the virus multiplied in susceptible cells of the lower alimentary tract and elicited development of specific neutralizing antibodies. The alimentary infection was independent of recognizable illness. Antibodies present in colostrum prevented alimentary infection during the first few weeks of life, but thereafter immunity was reinforced as a result of active infection.

Swine enterovirus was dispersed widely in pigs and in their habitat at the Municipal Farm at Kansas City, Mo. Other pigs in herds located elsewhere in Missouri provided serologic evidence of past infection. The virus undoubtedly spreads from pig to pig by direct fecal contamination. Houseflies *(Musca domestica)* undoubtedly serve as vectors in transmission, but, in an environment where most young pigs are infected, flies do not seem necessary for conveyance of virus from pig to pig.

References

[1] Beran, G. W., Werder, A. A., and Wenner, H. A.: Enteroviruses of Swine. I. Their Recognition, Identification, and Distribution in a Herd of Swine. Am. J. Vet. Res., 19, (July, 1958): 545–553.

[2] Beran, G. W., Wenner, H. A., Werder, A. A., and Underdahl, N. R.: Enteroviruses of Swine. III. Studies on Experimental Infection in Pigs. Am. J. Vet. Res., 21, (Sept., 1960): In press.

[3] Betts, A. O.: Respiratory Diseases of Pigs. V. Some Clinical and Epidemiological Aspects of Virus Pneumonia of Pigs. Vet. Rec., 64, (1952): 283–288.

[4] Committee on the Enteroviruses, National Foundation for Infantile Paralysis: The Enteroviruses. Am. J. Pub. Health, 47, (1957): 1556–1566.

[5] Dulbecco, R., and Vogt., M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., 99, (1954): 167–182.

[6] Li, C. P., and Schaeffer, M.: Serum Neutralization Tests in Mice and in Tissue Culture Against Three Types of Poliomyelitis Virus. J. Immunol., 72, (1954): 123–130.

[7] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

[8] Young, G. A., Underdahl, N. R., and Hinz, R. W.: Procurement of Baby Pigs by Hysterectomy. Am. J. Vet. Res., 16, (Jan., 1955): 123–131.

[9] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersd Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., 85, (1954): 202–205.

SUMMARIO IN INTERLINGUA

Enterovirus Porcin. II. Studios del Historia Natural de Infection e Immuniate

Enterovirus porcin (racia MF, i.e. ferma municipal) es un agente infectiose. In juvene porcos, le virus multiplicava in cellulas sueceptibile in le vias infero-alimentari e evocava le disveloppamento de un specific anticorpore neutralisante. Le infection alimentari esseva independente de recognoscibile formas de maladia. Anticorpore presente in le colostro preveniva un infection alimentari durante le prime septimanas del vita, sed postea le immunitate esseva reinfortiate como resultato de infection active.

Enterovirus porcin esseva dispergite extensemente inter le porcos al Ferma Municipal de Kansas City, Missouri, e in lor habitat. Altere porcos, ab greges locate alterubi in Missouri, provideva indicationes de infection passate. Le virus se propagava sin dubita ab un porco al altere per contamination fecal directe. Le musca domestic servi certemente como vector de transmission, sed in un ambiente in que le majoritate del juvene porcos es inficite, muscas non pare esser necessari pro le transmission del virus ab un porco al altere.

Detection of Swine Nasal Viruses in Cell Cultures

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THE DEVELOPMENT of cell culture systems affords an opportunity to detect, propagate, and study hitherto unknown swine viruses. The work described here was undertaken to determine if unknown viruses could be detected in swine nasal cavities. It is the purpose of this report to present available information about such viruses isolated from swine nasal cavities.

METHODS AND MATERIALS

The cell culture techniques used in this work were previously described by Switzer.[5] Nasal swabs were collected from pigs entered for evaluation studies at a testing station and from a few selected pigs submitted to the Iowa Veterinary Diagnostic Laboratory. Mild coughing and sneezing were evidenced by some of these pigs. The pigs came from many different areas of Iowa. Four pigs from each herd were housed in individual pens at the testing station. Nasal swabs were collected from 2 of the 4 pigs and pooled as a single sample. The specimens were collected after the pigs had been in the concentration area for a few days to several weeks.

The nasal swabs were returned to the laboratory within two hours after collection and vigorously swirled in 2-ml. of saline solution as suggested by Marcus *et al.*[3] This fluid contained 1,000 units of penicillin, 2,000 μg. of streptomycin, 1,000 units of nystatin,* and 250 μg. of erythromycin per milliliter. Erythromycin was included to inhibit *Mycoplasma hyorhinis*. The fluid was then frozen at least one hour at −20 C. to destroy or devitalize nasal trichomonads. It was thawed and allowed to stand at room temperature for two hours, then centrifuged at 2,000 r.p.m. for 15 minutes. Supernatant fluid (0.1 ml.) was inoculated into each of four cell culture tubes. The cell cultures were observed for five days or until definite cell damage occurred. All cell culture fluids were passaged a second time before being considered negative. Serial passage swine kidney and swine nasal mucosa cell lines were utilized initially but were discarded in favor of primary swine kidney cell cultures when satisfactory virus recovery and propagation was demonstrated for the latter.

Ethyl ether inactivation trials were conducted, utilizing the technique described by Andrewes.[1]

The ability of the various isolates to hemagglutinate a 10 per cent suspension of chicken red blood cells and 5 per cent suspensions of guinea pig, dog, rat, and mouse red blood cells at pH 7.0 at room temperature was determined by the plate method.

Carworth Farms CFW mice were inoculated at less than 48 hours of age by intracranial and subcutaneous routes to determine the action of these viruses on young mice. The mice were observed for three weeks.

Young adult rabbits of mixed breeding, secured from a local dealer, were inoculated subcutaneously with 2 ml. of virus material, and observed one month.

Six- to 8-day chicken embryos were inoculated by the yolk sac route, and 11-day chicken embryos were inoculated by the chorioallantoic sac route with the various viruses. Materials were harvested on the seventh day postinoculation and were passaged a second time before being considered negative.

Fluids from all suspected viral isolates were cultured in ox heart infusion-avian serum medium,[4] in NIH thioglycolate broth, and on horse blood-tryptose blood agar base medium.

Giemsa-stained, cover slip, cell-culture preparations infected with the suspected viral isolates were prepared by procedures described previously.[5]

RESULTS

Nasal swabs were collected from swine originating from 76 Iowa herds. Cytopathogenic agents, isolated by means of cell culture from pigs from 12 of these herds, were believed to be viruses. They passed Selas No. 02 filters, did not grow in ox heart infusion-avian serum medium, NIH thioglycolate broth, or on horse blood agar. However, examination of Giemsa-stained cover slip preparations of cells infected with various of the cytopathogenic isolates revealed that cells infected with two of these isolates (6 and 8) contained large numbers of coccobacillary bodies within the cytoplasm. These proved to be pleuropneumonia-like organisms, requiring additional serial passages to produce detectable growth in ox heart infusion-avian serum medium.*

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The technical assistance of Arlene Cole is gratefully acknowledged.

* Mycostatin, produced by E. R. Squibb & Sons, New York, N.Y.

* Appreciation is expressed to Dr. R. Ross of this laboratory for these additional cultivation attempts.

TABLE 1—Certain Properties of Viral Isolates Obtained from Swine Nasal Cavities

Properties	Virus No.										
	1	2	3	4	5	6	7	8	9	10	11
Hemagglutination of chicken, guinea pig, dog, rat, and mouse r.b.c.	—	—	—	—	—	—	—	—	—	—	—
Effects on chicken embryos											
chorioallantoic sac route	—	—	—	—	—	—	—	ND	—	—	—
yolk sac route	—	—	—	—	—	+	—	ND	—	—	—
20% ethyl ether susceptibility	—	—	—	—	—	+	—	+	—	—	—
Filtration through Selas 02 filter	+	+	+	+	+	+	+	+	+	—	—
Effect on mice	—	—	—	—	—	—	—	—	—	—	—
Effect on rabbits	—	—	—	—	—	—	—	—	—	—	—
Type of cell destruction	3*	3	3	2	2	1	3	1	3	3	2

— no effect; + effect; ND = not done. * *See* text for description of type of cell destruction.

TABLE 2—Survival of Two Swine Nasal Viruses at —20 C.

Isolate No.	Days stored at —20 C.		
	1	9	63
4	$10^{2.7}$ *	$10^{4.5}$	$10^{2.5}$
6	$10^{5.7}$	$10^{5.5}$	$10^{3.3}$

* Cell culture infective doses of virus per milliliter, based on 50 per cent end point.

Although 12 cytopathogenic isolates were obtained from this series of samples, one failed to produce detectable cell damage beyond the fourth serial passage. The 11 remaining isolates have been successfully passaged nine to 18 times. Certain of their properties have been determined. The hemagglutination ability of these 11 isolates; their ethyl ether resistance; their filterability; and their pathogenicity for chicken embryos, young mice, and rabbits are summarized (table 1).

None of these isolates hemagglutinated any of the species of red blood cells tested. They all passed Selas No. 02 filters and produced no detectable illness in mice or rabbits. The two isolates that were completely inactivated by 20 per cent ethyl ether produced a similar type of cell degeneration and were shown to be pleuropneumonia-like organisms. The titer of none of the other isolates was decreased by the ethyl ether treatment.

TABLE 3—Virus Titer in Fluids from Cell Cultures Infeted with Swine Nasal Virus Isolates

Isolate	Titer range	No. of virus passages titered
1	$10^{4.7}$–$10^{5.2}$*	4
2	$10^{4.0}$–$10^{5.0}$	6
3	$10^{2.7}$–$10^{5.0}$	4
4	$10^{2.5}$–$10^{4.5}$	8
5	$10^{2.7}$–$10^{3.7}$	4
6	$10^{3.3}$–$10^{6.0}$	9
7	$10^{3.5}$–$10^{5.2}$	4
8	$10^{4.3}$–$10^{5.7}$	4
9	$10^{3.5}$–$10^{6.8}$	5
10	$10^{3.7}$–$10^{5.5}$	4
11	$10^{3.0}$–$10^{4.2}$	3

* Cell culture infective doses per milliliter, based on 50 per cent end point.

Cell-culture fluids containing the various isolates were inoculated into chicken embryos. Isolate 6, inoculated by the yolk sac route, produced death in 2 of 6 chicken embryos on first passage and in 4 of 6 chicken embryos on second passage. The mortality was characterized by hemorrhage of the embryo. None of the other isolates produced embryo mortality, but chorioallantoic sac fluids from the second chicken embryo passage of isolates 2 and 3 produced typical cytopathic effects in cell cultures. Titrations of these fluids were not performed.

It was observed that all of the viruses isolated in this series produced definite cell destruction within five days in at least one cell culture tube on initial passage. Cell destruction initially appeared three to five days postinoculation, but on subsequent passages it appeared in two to four days. Microscopic examination of the cells undergoing destruction indicated that three different types of cell degeneration were produced by the 11 isolates.

Type 1 cell damage produced an intensely granular cytoplasm with subsequent cytoplasmic extrusions and fragmentation which give the cell a feathery appearance. The affected cells were yellowish brown and the cell sheet became a loose network of damaged and disintegrating cells. The nucleus appeared to be involved after the cytoplasm and became pyknotic. This damage appeared at the base of the cell sheet and spread toward the apex (fig. 1).

Type 2 cell damage was characterized by mild rounding of the affected cells, which become very granular. This was followed by disintegration of the cytoplasm and nucleus with formation of some cytoplasmic

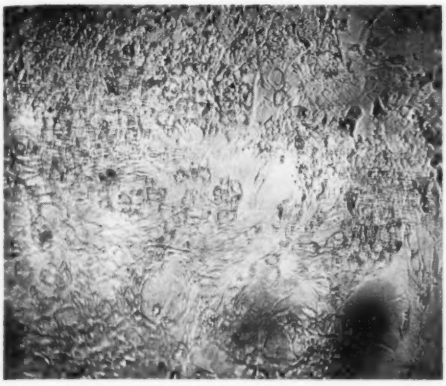

Fig. 1—Type 1 cell destruction in an unstained primary swine kidney cell sheet on the inner surface of a 16- by 150-mm. tube. Notice the intensely granular cell cytoplasm. x 33.

Fig. 2—Type 2 cell destruction in an unstained primary swine kidney cell sheet on the inner surface of a 16- by 150-mm. tube. There are numerous extruded fragments of cytoplasm surrounding some cells. The cells become granular and usually do not clump together. x 33.

strands. Cell destruction first involved the middle or apex portion of the cell sheet (fig. 2).

Type 3 cell degeneration consisted of initial rounding of the cells, which were clear and refractive, having undergone a two or threefold increase in cell volume. The cells superficially resembled coccidial oocysts. This stage was followed by disintegration of the cytoplasm and nucleus with some clumping of the cells and comparatively rapid release of the affected cell from the glass surface. This type of cell destruction usually affected the middle or apex portion of the cell sheet first (fig. 3).

Information was obtained on the storage of virus isolates 4 and 6 at −20 C. A pool of infected cell culture fluid was collected, kept frozen for one day, thawed, titrated, and dispensed in 2-ml. amounts in 16- by 150-mm. rubber-stoppered tubes, and refrozen at −20 C. The vials were thawed prior to preparation of tenfold dilutions utilizing Dulbecco's [2] phosphate buffer plus 10 per cent calf serum as diluent. The effect of length of storage on the virus titer of these samples is given (table 2). It is possible that the second freeze-thaw cycle liberated additional virus particles of isolate 4, which resulted in the higher nine-day storage titer.

The virus titers obtained with the different isolates in cell culture fluid three to five days after inoculation are given (table 3). In most cases, this fluid was subjected

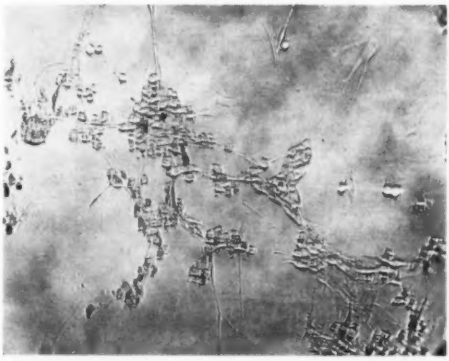

Fig. 3—Type 3 cell destruction in an unstained primary swine kidney cell sheet on the inner surface of a 16- by 150-mm. tube. The affected cells become refractile and clump together. x 33.

Fig. 4—Uninoculated, unstained primary swine kidney cell sheet on the inner surface of a 16- by 15-mm. tube. x 33.

TABLE 1—Certain Properties of Viral Isolates Obtained from Swine Nasal Cavities

Properties	Virus No.										
	1	2	3	4	5	6	7	8	9	10	11
Hemagglutination of chicken, guinea pig, dog, rat, and mouse r.b.c.	—	—	—	—	—	—	—	—	—	—	—
Effects on chicken embryos											
chorioallantoic sac route	—	—	—	—	—	—	—	ND	—	—	—
yolk sac route	—	—	—	—	—	+	—	ND	—	—	—
20% ethyl ether susceptibility	—	—	—	—	—	+	—	+	—	—	—
Filtration through Selas 02 filter	+	+	+	+	+	+	+	+	+	—	—
Effect on mice	—	—	—	—	—	—	—	—	—	—	—
Effect on rabbits	—	—	—	—	—	—	—	—	—	—	—
Type of cell destruction	3*	3	3	2	2	1	3	1	3	3	2

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6	$10^{3.3}$–$10^{6.0}$	9
7	$10^{3.5}$–$10^{5.2}$	4
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9	$10^{3.5}$–$10^{6.8}$	5
10	$10^{3.7}$–$10^{5.5}$	4
11	$10^{3.0}$–$10^{4.2}$	3

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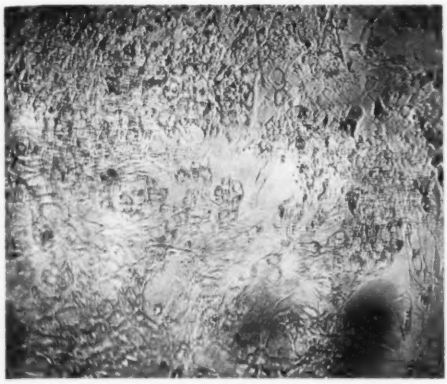

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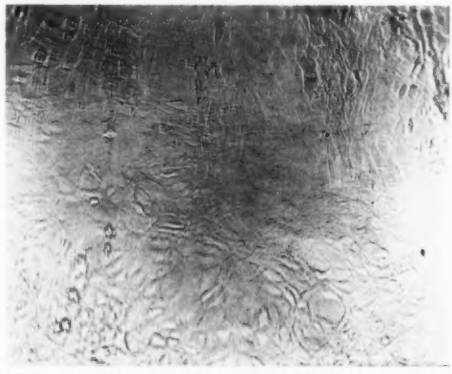

Fig. 4—Uninoculated, unstained primary swine kidney cell sheet on the inner surface of a 16- by 15-mm. tube. x 33.

to one freeze-thaw cycle of −20 C followed by room temperature or 37 C.

DISCUSSION

The recovery of viruses from the nasal cavities of swine does not establish whether they are inhabitants or contaminants.

The failure of any of the swine nasal virus isolates to produce disease in rabbits indicates that these viruses are not pseudorabies virus. In addition, it was observed that two different isolates of pseudorabies virus produced a somewhat different pattern of cell destruction in the cell culture systems used in this work.

Results obtained indicate that these virus isolates are not swine influenza virus. Propagation of known swine influenza virus strains in these cell culture systems has yielded a somewhat different type of cell destruction. In addition, it has been observed that swine influenza virus propagated in cell culture produces death in chicken embryos inoculated by the chorioallantoic sac route. The failure of any of these virus isolates to produce illness or death in suckling mice indicates they are not members of the Coxsackie group of viruses. Our experience indicates that the agent or agents responsible for a chronic transmissible pneumonia of swine, believed to be similar to virus pneumonia of pigs (VPP), does not multiply or produce cell destruction in the cell systems used in this work. Therefore, it appears that the group of virus isolates described are not pseudorabies virus, swine influenza virus, Coxsackie virus, or VPP virus. Two of this group of isolates appear to be a pleuropneumonia-like organism with considerable ability to invade cells.

Two antigenically distinct swine enteric viruses we have isolated and currently have under study produce cell damage similar to that described as type 3. This indicates the need for additional work to establish the habitat of the viruses isolated from the nasal cavity.

SUMMARY

Nasal-swab material from pigs originating from 76 Iowa herds was processed in cell culture systems. Cytopathogenic agents were isolated from 12 of these herds and 11 of the isolates were studied. They produced three different types of cell destruction; did not produce detectable changes in suckling mice, chicken embryos, or rabbits; passed Selas 02 filters; and did not hemagglutinate chicken, mouse, guinea pig, dog, or rat red blood cells. They are believed to be distinct from Coxsackie virus, swine influenza virus, pseudorabies virus, or virus pneumonia of pigs. Two isolates were shown to be pleuropneumonia-like organisms.

References

[1] Andrewes, C. H., and Horstmann, D. M.: The Susceptibility of Viruses to Ethyl Ether. J. Gen. Microbiol., 3, (1949): 290–297.

[2] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines of Poliomyelitis Virus. J. Exptl. Med., 99, (1954): 167–182.

[3] Marcus, P. I., Ciecura, S. J., and Puck, T. T.: Clonal Growth *in Vitro* of Epithelial Cells from Normal Human Tissues. J. Exptl. Med., 104, (1956): 615–628.

[4] Switzer, W. P.: Studies on Infectious Atrophic Rhinitis. IV. Characterization of a Pleuropneumonia-Like Organism Isolated from the Nasal Cavities of Swine. Am. J. Vet. Res., 16, (July, 1955): 540–544.

[5] Switzer, W. P.: Action of Certain Viruses, *Mycoplasma hyorhinis* and Nasal Trichomonads on Swine Tissue Cultures. Am. J. Vet. Res., 20, (Nov., 1959): 1010–1019.

SUMMARIO IN INTERLINGUA

Le Detection de Porcin Virus Nasal in Culturationes de Cellulas

Material ab tampones nasal de porcos, proveniente ab 76 greges in Iowa, esseva processate in systemas de culturation cellular. Agentes cytopathogene esseva isolate ab 12 del greges, e 11 del isolatos esseva studiate. Illos produceva tres differente typos de destruction cellular; illos non produceva detegibile alterationes in muses tettante, in embryones de gallina, o in conilios; illos passava filtros Selas 02; e illos non effectuava le hemagglutination de erythrocytos de gallina, mus, porco de Indian, can, o ratto. Es opinate que illos non es identic con virus Coxsackie, virus de influenza porcin, virus de pseudorabie, o virus de pneumonia porcin virusal. Esseve trovate que duo del isolatos representava organismos pleuropneumonioide.

Factors Affecting the Production and Detection of Ornithosis Antibodies in Infected Turkeys

L. A. PAGE, Ph.D., and R. A. BANKOWSKI, D.V.M., Ph.D.

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THE METHODS presently used for the detection of ornithosis antibodies in the serums of infected turkeys are applied primarily in disease diagnosis and, secondarily, in determining the incidence of positive reactors among large groups of turkeys within a region or within certain flocks. Although three valid serologic methods [2, 5-7] exist for testing turkey anti-ornithosis serums, a significant problem remains in the interpretation to be placed on test results, particularly when the results are neither strongly positive nor conclusively negative. This applies to the results of serologic surveys on any group of birds where clinical or microbiologic evidence of infection is equivocal or not known.

It is the purpose of this report to describe some of the factors affecting the production and detection of ornithosis antibodies in infected turkeys and to interpret results of serologic tests in the light of these factors.

MATERIALS AND METHODS

Three serologic methods were used to test 623 serum samples from 216 turkeys in various stages of experimental infection. The serologic responses to infection were tabulated according to the method employed to detect antibodies; the dosage, route, and strain of organisms inoculated; and the time after inoculation when the serums were collected. Where necropsy data were available, the appearance of gross lesions in infected birds was correlated with the serologic response and the inoculation dose.

Test Animals.—Broad Breasted Bronze turkeys, 12 or more weeks old, were used in all infection experiments. All birds were serologically negative prior to each experiment. The number of ornithosis organisms in various inoculums was determined by titrating each suspension in white mice (Webster strain) according to the methods previously reported.[10] Calculation of 50 per cent infectivity end points was based on the method of Reed and Muench.[14]

Strains of Ornithosis Organisms.—All strains used in the experiments were isolated from naturally infected turkeys and pigeons in California. The C-1, C-2, and C-4 strains were isolated during epornities in turkeys in 1954, 1956, and 1958.[1, 12] The CP-1 and CP-3 strains were isolated from pigeons in 1956 and 1958. Suspensions of these agents were prepared as inoculums by grinding infected mouse spleens and livers in a mortar to make a 20 per cent tissue suspension in beef heart infusion broth.

The Complement-Fixation Inhibition Test.—The principal serologic test for detecting ornithosis antibodies in turkey and chicken serums is the complement-fixation inhibition (CFI) test of Karrer *et. al.*,[6] as modified by Meyer and Eddie.[8] The antigen used in the CFI tests referred to herein was lot 13 prepared by R. W. Moore, Texas Agricultural Experiment Station. It consisted of yolk sac-propagated 6BC strain of *Miyagawanella psittacii*[4] which had been suspended in beef heart broth, heated for 30 minutes at 100 C., and preserved with 0.5 per cent phenol.

The CFI procedure was conducted as recommended by Meyer and Eddie,[8] except for the determination of the optimum dilutions of antigen and indicator antibody (human anti-ornithosis serum) to be used in the test. For this purpose, a box titration of the CFI antigen against indicator antibody (lot VZ) was performed. The titration revealed the highest dilutions of antigen and indicator serum at which complete fixation of 2 exact units of complement occurred (table 1) and thereby defined the unitage of each reactant. Use of these dilutions in the CFI procedure produced a reproducible test; whereas, in our hands, use of antigen and serum dilutions slightly higher than those producing complete complement fixation as suggested by Meyer and Eddie[8] resulted in too great an increase in the sensitivity of the test.

The titer of each turkey serum sample was defined as the reciprocal of the highest dilution of serum that caused complete complement-fixation inhibition in the presence of 1 unit of antigen, 1 unit of indicator serum, and 2 exact units of complement using the volumes listed. With some turkey serums, partial CF inhibition occurred in the tubes in which a reaction was apparent. Titers of these serums were designated as the highest dilution of serum with a visual two-plus (++) reaction expressed, e.g., as p32, or partial to a dilution of 1:32. All CFI tests were incubated for 120 minutes at 37 C. prior to addition of sensitized sheep red blood cells (r.b.c.) and for 60 minutes at 37 C.

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after r.b.c. addition to insure maximum sensitivity (table 1).

Direct Complement-Fixation Test.—Benedict and O'Brien [3] developed a soluble antigen by extracting the meningopneumonitis strain of *Miyagawanella ornithosis* [4] with sodium lauryl sulfate. The antigen fixed complement in the presence of heat-inactivated anti-ornithosis serum, avian or mammalian. A direct complement-fixation (DCF) technique was used.[2] The DCF antigen used was obtained from A. A. Benedict, University of Kansas. One unit of antigenic activity of the preparation was defined as the highest dilution of antigen which caused the complete fixation of 2 exact units of complement in the presence of 2 units of heat-inactivated turkey anti-ornithosis serum using the volumes listed. Anti-ornithosis titers of turkey serums were expressed as the reciprocal of the highest dilution of serum causing complete fixation of 2 exact units of complement with 2 units of antigen. All DCF test serums were incubated for 90 minutes at 37 C. prior to addition of sensitized sheep r.b.c. and for 30 minutes at 37 C. after r.b.c. addition, since a longer pre-r.b.c. incubation period (table 1) reduced the sensitivity of the test.

Capillary Tube Agglutination Test.—Mason [7] developed an agglutination test utilizing a purified, stained concentrate of elementary bodies of the NJ-1 turkey strain [11] of *M. ornithosis* as antigen. To perform this capillary tube agglutination (CTA) test, the antigen was drawn by capillary attraction to half the length of a glass tube, 100 mm. long with a bore of 0.4 mm. The capillary tube was then dipped into unheated test serum diluted 1:8

TABLE 1—Effect of Serum Inactivation and Incubation Time Variation on Standardization of the Direct Complement-Fixation and Complement-Fixation Inhibition Antigens by Complement-Fixation Tests

Incubation time (min.) at 37 C.					DCF					CFI*				
		Complement dilution for 2 exact units	Ornithosis antiserum	Reciprocal dilution serum	Antigen dilution					Antigen dilution				
Pre-r.b.c.	Post-r.b.c.				8	16	32	64	128	128	256	512	1,024	2,048
120	60	31.2	Turkey, lot 156. Heated 56 C., 30 min.	16	4	3	1	±	0	0	0	0	0	0
				32	3	3	±	±	0	0	0	0	0	0
				64	1	±	±	0	0	2	0	0	0	0
				128	0	0	0	0	0	4	2	0	0	0
				256	0	0	0	0	0	4	3	0	0	0
				512	0	0	0	0	0	4	3+	±	0	0
				1,024	0	0	0	0	0	4	3	2	±	0
				2,048	0	0	0	0	0	4	2	1	0	0
			Not heated	16	1	±	0	0	0	±	±	0	0	0
				32	3	±	0	0	0	±	0	0	0	0
				64	±	±	0	0	0	2	0	0	0	0
				128	±	±	±	0	0	4	±	0	0	0
				256	±	±	±	±	±	4	2	1	0	0
				512	±	±	±	±	±	4	3	±	±	±
				1,024	±	±	±	±	±	4	3+	2	1	±
				2,048	±	±	±	±	±	4	3	±	±	±
90	30	37.5	Heated 56 C., 30 min.	16	4	(4)**	2	±	0	0	0	0	0	0
				32	3+	3	2	±	±	±	0	0	0	0
				64	2	3	2	±	±	2	±	0	0	0
				128	0	0	0	0	0	3	2	±	0	0
				256	0	0	0	0	0	3	2	±	0	0
				512	0	0	0	0	0	4	2	±	0	0
				1,024	0	0	0	0	0	4	3	1	0	0
				2,048	0	0	0	0	0	4	3	1	0	0
120	60	31.2	Human, lot VZ. Heated 56 C., 30 min.	64	4	3+	1	0	0	4	4	3+	1	0
				128	4	3+	±	0	0	4	4	3	±	0
				256	3+	2	±	0	0	4	(4)	±	0	0
				512	2	±	0	0	0	1	±	0	0	0
				1,024	0	0	0	0	0	0	0	0	0	0
			Not heated	64	4	3+	1	±	±	4	4	3+	1	0
				128	4	3+	±	±	±	4	4	3	±	0
				256	4	2	±	0	0	4	(4)	2	±	0
				512	2	±	0	0	0	3+	2	±	0	0
				1,024	±	0	0	0	0	1	±	0	0	0
90	30	37.5	Heated 56 C., 30 min.	64	4	4	2	0	0	4	4	3+	3	0
				128	4	3+	±	0	0	4	4	3+	2	0
				256	3+	3	±	0	0	4	4	3	1	0
				512	2	1	0	0	0	4	4	1	±	0
				1,024	±	±	0	0	0	4	3+	1	±	0

All reactants were diluted in veronal-buffered saline (pH 7.2) and added to tubes in 0.25-ml. amounts. All readings are in terms of complement fixation.

* One unit of indicator serum, lot VZ, was added to each tube in CFI tests on turkey serum, but not to tubes in tests on human lot VZ, serum.

** The reaction which represents complete fixation of complement at the highest dilutions of antigen and antiserum is shown in parentheses.

or more with veronal-buffered saline solution until the tube was filled. The filled tube was then forced a short distance into a block of plasticine or clay to insure that the antigen remained above the serum in the capillary column. A separate tube of antigen was used for each dilution of serum.

In the presence of turkey anti-ornithosis serum, the blue antigen formed readily visible clumps in the capillary column within four hours at room temperature; however, final readings were made after 18 to 24 hours. Reactions were most distinct when observed against a white background. Tubes containing visible clumps were considered positive. Accumulations of very fine aggregates of particles along the capillary column were doubtful reactions and were disregarded in determining the titer of the serum. Tubes with a clear column were read as negative reactions. The antigen used was lot 1, prepared by D. M. Mason, George Williams Hooper Foundation for Medical Research, San Francisco, Calif.

General Procedure for All Serologic Tests.—The activity of all antigens was standardized periodically against turkey (lot 156) and human (lot VZ) anti-ornithosis serums. Antiserums of lots 156 and 151, derived from turkeys experimentally infected with the NJ-1 ornithosis agent, and normal turkey serum, lot A-1, were used as positive and negative control serums in daily CFI, DCF, and CTA tests. Lot VZ serum, used as indicator antibody in the CFI test, was obtained in 1957 from a person who became afflicted with ornithosis after associating with diseased turkeys in Oregon.

To minimize errors, all three serologic tests on any group of turkey serums were performed on the same day; and, whenever possible, all serums collected at intervals from any 1 bird were tested on the same day. For the comparative tests, serial twofold dilutions of turkey test and control serums, beginning with 1:8, were made in triplicate in veronal-buffered saline solution (pH 7.2). Two of the sets of dilutions were inactivated at 56 C. for 30 minutes prior to use in the DCF and CFI tests; a third set, unheated, was used in the CTA test. As indicated (table 1), failure to heat the turkey serums reduced the sensitivity of the complement-fixation tests. Preliminary readings of the CF reaction systems were made after final incubation at 37 C. Final readings were made after 18 hours at 6 C.

TABLE 2—Results of Capillary Tube Agglutination, Complement-Fixation Inhibition, and Direct Complement Fixation Tests on Serums Collected from Turkeys on Consecutive Days After Inoculation with the C-1 Ornithosis Agent

Days after inoculation	Reciprocal titers								
	100,000 mouse $i.d._{50}$ intravenously						3,000 mouse $i.d._{50}$ intratracheally		
	Turkey 3295			Turkey 3294			(turkey 3298)		
	CTA	CFI	DCF	CTA	CFI	DCF	CTA	CFI	DCF
0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0			
2	0	0	0	0	0	0			
3	4	0	0	0	0	0	0	0	0
4	8	0	0	0	0	0			
5							0	0	0
6	16	0	0	0	0	0			
7	16	0	0	0	0	0	0	0	256
8	16	4	4	0	0	0			
9	16	32	8	0	0	p4	0	0	>1,024
10	64	128	64	0	0	512			
11	64	256	256	8	0	256			
12	64	512	256	16	0	4			
13	128	256	512	8	0	p4	0	p8	>1,024
14	128	512	128	0	0	p16			
15							16	32	>1,024
16	128	512	256	0	p8	p8			
20							32	128	>1,024
22	32	512	128	0	0	16			

The turkeys used in this experiment were 9 months of age.

All reactants in the DCF and CFI tests were added to each tube in 0.25-ml. amounts, except that the suspension of 2 per cent sheep r.b.c., combined with hemolysis, was added in 0.5-ml. amounts. Final volume in all CF tests was 1.5 ml.

RESULTS

To determine the order of appearance of antibodies detectable by the CFI, DCF, and CTA methods, 2 normal turkeys were inoculated intravenously with 100,000 mouse $i.d._{50}$ of ornithosis organisms of the C-1 strain, and blood samples were collected daily for serologic testing. Blood samples from another bird, inoculated intratracheally with 3,000 mouse $i.d._{50}$ of the same strain, were obtained at irregular

intervals for three weeks. The results of these tests (table 2) confirmed the implication of previous experiments that antibodies detectable by the CTA and DCF methods appeared before antibodies demonstrable by the CFI method in the serums of turkeys inoculated with ornithosis organisms. Agglutinins appeared in the serum of 1 turkey as early as three days after an intravenous dose of live organisms, preceding the DCF and CFI antibodies by five days. In the second turkey which was inoculated intravenously, DCF antibodies appeared on the ninth day after inoculation, preceding the demonstrable agglutinins by two days and the CFI antibodies by seven days. In the bird inoculated intratracheally, DCF antibodies appeared in high titer at least six days before CFI or CTA antibodies.

The 330 serum samples collected at intervals from three groups of turkeys inoculated intraperitoneally with 3,000 mouse $i.d._{50}$ of the C-1, C-2, or CP-3 ornithosis agents were also tested by the CFI, DCF, and CTA methods. Serums were collected from each bird on the nineteenth, 46th, and 104th day after inoculation. During this period, the stage of ornithosis infection in the flock was determined at monthly intervals by necropsy of representative numbers of birds from each group and by attempts on each sampling to isolate ornithosis agents. In the serologic findings (table 3) it was seen that three factors markedly influenced the production and detection of ornithosis antibodies in infected turkeys: (1) the strain of organism used for infection; (2) the serologic method used; and (3) the number of days after infection that the serum was collected.

From these data, it was clear that the CTA and DCF tests had greater sensitivity for detecting antibodies produced early in the course of ornithosis than the CFI test. A corresponding high degree of agreement, *i.e.*, 93.3 per cent of the serums were positive (or negative) by the CTA and DCF tests, existed between the two tests on the nineteenth day after inoculation, or in the later stage of the disease, more of the birds developed antibodies that were detected by the CFI method, and the agreement among the three methods improved. The CFI, DCF, and CTA tests of serums collected on the 104th day after inoculation, when the infected birds were shown to have recovered from ornithosis, were in poor agreement; *i.e.*, only 40.7 per cent of the serums were positive (or negative) by all three of the serologic methods. By this time, many birds that had CTA titers on the nineteenth or 46th day had no CTA titer. For the most part, CFI or DCF titers persisted longer than CTA titers in infected birds.

TABLE 3—Tabulation of Results of Capillary Tube Agglutination, Complement-Fixation Inhibition, and Direct Complement-Fixation Tests on 330 Serum Samples Collected at Intervals from Turkeys Experimentally Infected with Any of Three Strains of Ornithosis Agents

Test result			No. of serum samples on postinoculation* day: 19				46				104			
			Strain				Strain				Strain			
CTA	CFI	DCF	C-1	C-2	CP-3	%	C-1	C-2	CP-3	%	C-1	C-2	CP-3	%
+	+	+	20	14	16	42.0	26	20	32	69.0	6	12	9	26.5
−	−	−	0	13	1	11.8	0	11	0	9.8	5	7	2	14.2
+	+	−	0	0	0	0	2	0	0	1.8	1	1	5	7.1
−	−	+	0	2	4	5.0	1	2	3	5.3	6	2	5	13.3
+	−	+	19	11	17	39.5	7	2	2	9.8	2	0	1	3.0
−	+	−	0	0	0	0	0	0	0	0	5	5	4	14.4
−	+	+	0	0	2	1.7	0	1	1	1.8	6	4	7	17.4
+	−	−	0	0	0	0	1	2	0	2.5	0	3	1	2.1
Total serum			119			100	113			100	98			100

Tests	Per cent agreement among serologic tests at various stages of disease: Acute (19 days)	Convalescent (46 days)	Recovered (104 days)	Combined
CTA–CFI–DCF	53.8	78.8	40.7	58.5
CTA–CFI	58.8	86.0	61.1	69.0
CTA–DCF	93.3	88.6	58.5	81.1
DCF–CFI	55.5	83.2	62.1	66.9

* Inoculation with 3,000 mouse $i.d._{50}$.

A marked difference in the ability of the two turkey ornithosis strains (C-1 and C-2) to stimulate antibody production was apparent. On the nineteenth day after inoculation, 100 per cent of the birds inoculated with 3,000 mouse i.d.$_{50}$ of C-1 organisms had demonstrable antibodies in their bloodstreams; whereas, only 67 per cent of the birds inoculated with an identical dose of the C-2 strain developed demonstrable antibodies by the nineteenth day. By the 46th day, 97 per cent of the C-1 inoculated birds were still positive and 74 per cent of the C-2 inoculated birds were positive by any of the serological methods. The percentage of reactors among the birds inoculated with the pigeon ornithosis agent (CP-3) was similar to that of those given the C-1 strain, although several of the birds in the CP-3 group developed antibodies demonstrable by the CFI method prior to development of antibodies detected by the other methods. This confirmed a previous report by Page.[13]

The correlation between the serologic and pathologic response, as well as the effect of varying the dose on these responses, is shown (table 4) for turkeys experimentally inoculated with the C-2 strain. Agglutinins and CFI antibodies were readily detected in the serums of turkeys two weeks after the birds were inoculated intraperitoneally with 43 or more i.d.$_{50}$ of the C-2 strain, and a positive serologic response was reflected in most cases by the appearance, at necropsy, of gross lesions typical of ornithosis as described previously.[1, 11, 13] Low CTA titers, negative CFI titers, and absence of lesions resulted from the birds being inoculated with smaller doses of the C-2 strain. Individual resistance to infection, as indicated by failure to develop antibodies or gross lesions, was observed in every inoculation group.

A similar study with the C-4 strain indicated that this organism was less infectious and less antigenic than the C-2 strain. Intraperitoneal inoculation of 6,800 mouse i.d.$_{50}$ (but not 68 mouse i.d.$_{50}$) produced severe lesions of ornithosis and CTA titers within two weeks in 4 of 5 inoculated birds. Antibodies were not detected in these birds by the CFI test. Results of the titration of the C-2 and C-4 strains in turkeys indicated that the serologic and pathologic response depended not only on the dose but also on the strain of organisms employed.

Serologic findings (determined by the CTA and CFI methods) from all of the experimental ornithosis trials conducted to date at this laboratory are summarized (table 5). At the time some of these trials were conducted, the DCF antigen was not available; therefore, comparison is made only between results of CFI and CTA tests. A total of 839 serums from 216 turkeys was tested; 623 serums were collected after experimental ornithosis infection, and 216 were collected prior to inoculation. All 216 birds were serologically negative before infection.

Since 22.4 per cent of the serums collected from turkeys after inoculation with ornithosis agents were CTA positive and CFI negative, it was evident that the agglutination was more sensitive than the CFI test for detecting ornithosis antibodies. Part

TABLE 4—Serologic and Pathologic Response of Turkeys to Intraperitoneal Inoculation of Varying Doses of C-2 Turkey Ornithosis Agent

Turkey No.	Dose (mouse i.d.$_{50}$)	Reciprocal titers 14 days after inoculation*		Degree of gross lesions** 14 days after inoculation
		CTA	CFI	
574	430	32	16	++++
575	430	64	p32	++++
576	430	16	16	++++
577	430	32	16	++++
578	430	16	0	+
579	Pen contact	8	p8	+
580	Pen contact	0	0	+
581	43	32	32	++++
582	43	32	32	++++
583	43	0	0	0
584	43	16	8	++++
585	43	64	32	++++
586	Pen contact	0	0	0
587	Pen contact	0	0	0
588	4	32	8	+
589	4	0	0	0
590	4	32	0	++
591	4	0	0	0
592	4	32	32	++
593	Pen contact	0	0	0
594	Pen contact	0	0	0
595	<1	8	0	0
596	<1	0	0	0
597	<1	0	0	0
598	<1	0	0	0
599	<1	0	0	0
600	Pen contact	0	0	0
.....	Pen contact	0	0	0

* All turkeys were serologically negative before inoculation. ** + = fibrinous myocardial plaque; ++ = small fibrinous plaques on heart or liver, areas of localized aerosacculitis; +++ = fibrinous plaques on heart or liver, moderate peritonitis or aerosacculitis, slight splenomegaly; ++++ = extensive fibrinous pericarditis, perihepatitis, peritonitis, aerosacculitis; splenomegaly, hepatomegaly; splenic and hepatic mottling.

of the greater sensitivity was due to the fact that agglutinins appeared earlier than CFI antibodies in the serums of turkeys experimentally infected with turkey ornithosis agents; furthermore, most of the serums examined by both tests had been collected during the acute stage of ornithosis. In time most, although not all, turkeys that had recovered from ornithosis developed antibodies demonstrable by both tests.

turkeys infected with identical doses of similar organisms; therefore, the sixth factor, resistance to parasitic multiplication or inability to react in a certain time to a given antigenic stimulus, greatly influenced the degree of antibody response demonstrable with any of the serologic tests.

Since the serologic response of the turkey to ornithosis infection was influenced by the strain, route, and number of organisms inoculated, as well as individual resistance

TABLE 5—Summary of Capillary Tube Agglutination and Complement-Fixation Inhibition Test Results from All Trials

	Test results		No. of serum samples from turkeys infected with:						
			Turkey agents			Pigeon agents*			
	CTA	CFI	C-1	C-2	C-4	CP-1A & CP-1B	CP-1C	CP-3	Totals
Before inoculation	—	—	69	60	15	18	14	40	216**
After inoculation	+	+	76	79	0	32	9	62	258/623† (41.7%)
(includes consecutive	+	—	52	23	4	20	20	20	139/623 (22.4%)
samples from some	—	+	13	9	0	30	1	15	68/623 (10.7%)
turkeys)	—	—	42	41	11	37	13	14	158/623 (25.2%)

* The turkey serum samples listed in the column headed CP-1A and CP-1B represent samples collected from turkeys infected with the CP-1 pigeon ornithosis agent by inoculation or by contact with infected pigeons. The serums under CP-1C represent samples from turkeys exposed to ornithosis infection by contact with turkeys in the CP-1A or CP-1B groups. ** Represents the number of birds used in all trials. † 623 was the total number of serums collected after the turkeys were inoculated.

DISCUSSION

The serologic findings reported here represent results of tests on serums collected from turkeys maintained under a variety of controlled conditions of experimental infection; therefore, the results encompass response to a broad range of host-parasite relationships in ornithosis.

Marked differences were seen in the results of serologic testing of ornithosis-infected birds (tables 2–5). The differences were attributed to six factors: the serologic method used, the serum collection time, the strain of infectious agent used, the dose, the route of inoculation, and individual resistance to infection. Had strains or species of birds other than the Broad Breasted Bronze turkey been tested, genetically controlled factors would probably play a larger role in accounting for differences in serologic response.

It was reasonable to assume that the degree of antibody response to ornithosis agents depended upon the quality and quantity of antigenic material available at the antibody production sites, as well as upon the repetition of the antigenic stimulus. Resistance to infection varied among individual turkeys, as evidenced by the varying degree of gross lesions observed in

to infection, full agreement of the results of CFI, DCF, and CTA tests on any group of immune serums can never be achieved. The differences among the test methods and their respective antigens enlarge the range of disagreement. The use of several serologic methods with full knowledge of their different sensitivities may, however, be of some value in itself; use of the several tests may focus attention on biologic events which are quite possibly different aspects of the host-parasite relationship.[13]

Since all three serologic tests measure antibodies to ornithosis organisms, the choice of the one to use depends upon the facilities and resources of the individual poultry disease diagnostic laboratory. The usefulness of the CTA test lies in its simplicity of performance and in its applicability to screening large numbers of avian serums for the presence of ornithosis antibodies. This does not mean, however, that the other methods cannot be applied for serological screening.

Since it would be desirable to have a serologic test that correlated well with clinical or pathologic findings, a critical question arises: is the CTA (or DCF) test too sensitive? In the authors' opinion, the answer is no, since serologic tests measure

antibody response to infection and are not necessarily correlated with clinical disease; therefore, the tests must be interpreted in their proper perspective. A highly sensitive test for the early detection of ornithosis antibodies in poultry flocks is desirable inasmuch as diagnosis of the disease ultimately rests on isolation of the causative agent, not on the mere existence of antibody in one serum sample.

Neal and Davis[9] reported a 34.3 per cent correlation among results of CFI, DCF, and plate agglutination tests on 923 serums from ornithosis-infected and apparently normal turkeys. Their plate agglutination antigen differed from the CTA antigen in its preparation, strain of organism used as antigen, and in its use. Of 830 serums giving positive reaction by either the CFI, DCF, or plate agglutination tests, 712 were positive only in dilutions of 1:2 or 1:4, a range where nonspecific serum factors affect many serologic tests, particularly agglutination reactions. Agreement among results of the tests improved when the data on reactions at dilutions of 1:8 or more were correlated. Since the interval between initial exposure to ornithosis organisms and the day of collection of serum from the infected birds was not recorded, the authors could not determine the effect of time on the serologic response after infection, a factor which clearly affected the results reported herein.

SUMMARY

Ornithosis antibody titers of 623 serums collected from 216 turkeys experimentally infected with any one of five ornithosis agents were determined by the complement-fixation inhibition (CFI) test of Karrer *et al.*[6] and the capillary tube agglutination (CTA) test of Mason.[7] Of these serums, 370 were also tested by the direct complement fixation (DCF) test of Benedict.[2]

The presence of demonstrable antibodies in the serums of infected turkeys did not always correlate with the presence of gross lesions at necropsy. The serologic response varied with serologic method used to demonstrate antibodies; the number, route, and strain of organisms inoculated; the resistance of the host to infection; and the time after inoculation when the serum was collected.

In general, turkeys inoculated with turkey ornithosis agents developed antibodies demonstrable by the CTA and DCF tests early in the course of ornithosis and later became CFI positive. Antibodies demonstrable by DCF and CFI tests persisted longer in the bloodstreams of infected birds than did antibodies demonstrable by the CTA test. A proper interpretation of any serologic test result could not be made without consideration of these factors.

The results of CFI, DCF, and CTA tests on 330 serums collected at intervals from turkeys experimentally infected with three strains of ornithosis agents were compared. Percentage agreement of all three tests on serums taken on the nineteenth, 46th, and 104th days after inoculation were 53.8 per cent, 78.8 per cent, and 40.7 per cent, respectively.

For diagnostic purposes, tests for ornithosis antibodies in the serums of turkeys in flocks suspected of being affected with ornithosis should be used only to support further clinical, pathologic, and microbiologic investigations.

References

[1] Bankowski, R. A., and Page, L. A.: Studies of Two Epornitics of Ornithosis Caused by Agents of Low Virulence. Am. J. Vet. Res., 20, (Nov., 1959): 935–940.

[2] Benedict, A. A., and McFarland, C.: Direct Complement-Fixation Test for Diagnosis of Ornithosis in Turkeys. Proc. Soc. Exptl. Biol. & Med., 92, (1956): 768–771.

[3] Benedict, A. A., and O'Brien, E.: Antigenic Studies on the Psittacosis-Lymphogranuloma Venereum Group of Viruses. II. Characterization of Complement-Fixing Antigens Extracted with Sodium Lauryl Sulfate. J. Immunol., 76, (1956): 293–300.

[4] Bergey's Manual of Determinative Bacteriology. 7th ed. Williams & Wilkins Co., Baltimore, (1957): 961–968.

[5] Brumfield, H. P., and Pomeroy, B. S.: Direct Complement Fixation by Turkey and Chicken Serum in Viral Systems. Proc. Soc. Exptl. Biol. & Med., 94, (1957): 146–149.

[6] Karrer, H., Meyer, K. F., and Eddie, B.: The Complement-Fixation Inhibition Test and Its Application to the Diagnosis of Ornithosis in Chickens and Ducks. I. Principles and Techniques of the Test. J. Infect. Dis., 87, (1951): 13–23.

[7] Mason, D. M.: A Capillary Tube Agglutination Test for Detecting Antibodies Against Ornithosis in Turkey Serum. J. Immunol., 83, (1959): 661–666.

[8] Meyer, K. F., and Eddie, B.: Psittacosis. In "Diagnostic Procedures for Viral and Rickettsial Diseases." Pub. by Amer. Pub. Health Assoc., (1956): 399–430.

[9] Neal, J. W., and Davis, D. E.: A Comparison of the Indirect Complement-Fixation Test, the Direct Complement-Fixation Test, and the Macroscopic-Agglutination Test for Ornithosis Antibodies in Turkey Serums. Am. J. Vet. Res., 19, (Jan., 1958): 200–203.

[10] Page, L. A.: Measurement of Pathogenicity of Turkey Ornithosis Agents for Mice. Avian Dis., 3, (1959): 51–66.

[11] Page, L. A.: Experimental Ornithosis in Turkeys Avian Dis., 3, (1959): 51–66.

[12] Page, L. A., and Bankowski, R. A.: Investigation of a Recent Ornithosis Epornitic in California Turkeys. Am. J. Vet. Res., 20, (Nov., 1959): 941–945.
[13] Page, L. A.: Ecologic Considerations in Turkey Ornithosis. Am. J. Vet. Res., 21, (July, 1960): 618–623.
[14] Reed, C. J., and Muench, H.: A Simple Method for the Estimation of Fifty Percent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

SUMMARIO IN INTERLINGUA

Factores que Affice le Production e le Detection de Anticorpore Anti Ornithosis in Inficite Gallos de India

Le titros del anticorpore anti ornithosis esseva determinate in 623 seros colligite ab 216 gallos de India le quales habeva essite inficite experimentalmente per un del cinque agentes de ornithosis. Le tests usate esseva (1) le test del inhibition del fixation de complemento secundo Karrer *et al.* e (2) le test del agglutination in tubo capillari secundo Mason. Pro 370 del seros, etiam le test del fixation directe de complemento secundo Benedict esseva applicate.

Le presentia de demonstrabile concentrationes de anticorpore in le seros del inficite gallos de India non se correlationava in omne casos con le presentia de lesiones macroscopic al necropsia. Le responsa serologic variava con le methodo usate pro demonstrar le presentia de anticorpore. Illo etiam variava con le numero, le via de administration, e le racia del organismos inoculate, con le resistentia del hospite contra infectiones, e con le intervallo de tempore inter le inoculation e le collection del sero.

A generalmente parlar, gallos de India inoculate con agentes de ornithosis de gallos de India disveloppava tosto in le curso del ornithosis concentrationes de anticorpore demonstrabile per le test de agglutination a tubos capillari e le test directe del fixation de complemento, durante que positivitate per le test del inhibition de fixation de complemento esseva manifeste solmente plus tarde. Le anticorpore demonstrabile per le test directe del fixation de complemento e per le test del inhibition de fixation de complemento persisteva plus longemente in le circulation de inficite aves que le anticorpore demonstrabile per le test de agglutination a tubo capillari. Il non esserea possibile interpretar appropriatemente le resultatos de non importa qual test serologic si iste factores non es prendite in consideration.

Le resultatos del tres mentionate tests esseva comparate in applicationes parallel a 330 seros colligite a intervallos ab gallos de India inficite experimentalmente con tres racias de agentes de ornithosis. Le procentages de concordantia inter le tres tests in seros colligite 19, 46, e 104 dies post le inoculation esseva 53,8, 78,8 e 40,7, respectivemente.

Ab le puncto de vista diagnostic, tests pro anticorpore anti ornithosis in le sero de gallos de India in greges suspicite de esser afficite con ornithosis deberea esser usate solmente como supporto de altere investigationes clinic, pathologic, e microbiologic.

Pendulous Crops in Turkeys—an Anatomic and Pathologic Study

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PENDULOUS CROP has been attributed to the amount of protein in the ration [2] and to heredity.[3] Its occurrence has been observed more frequently in turkeys raised where the maximum air temperature and hours of sunshine are high and the relative humidity comparatively low.[1] Wheeler *et al.*, in 1957,[6] reported the experimental induction of pendulous crop in turkey poults fed a synthetic diet containing glucose* as the primary source of carbohydrates. Manfre *et al.*[4] demonstrated a fungus, *Saccharomyces tellustris,* in the crops of these poults and suggested that fermentation, resulting from this yeast, was the significant factor in the formation of these experimentally induced pendulous crops.

Although the anatomic appearance of experimentally induced pendulous crop in the turkey poult [5] is the same as that of the spontaneously acquired enlargement, it is likely that the cause of the two lesions is different. The present study has provided an anatomic demonstration and a histopathologic study of the muscles supporting the normal and the pendulous crop.

METHODS AND MATERIAL

Thirty-three Broad Breasted Bronze turkeys, varying in age from 10 weeks to 2 years, were used. Seventeen of these were normal birds. Of the 16 birds with pendulous crop, 7 were adult females weighing 9 to 17 lb., 1 was a male weighing 17 lb., and 3 were young poults weighing approximately 5½ lb. Five were adults, but the weight and sex were not recorded. Three of the birds with pendulous crops were obtained from a farm in California, 2 from Minnesota, 3 from Wisconsin, and 8 from the flock at the A. & M. College of Texas. Four of the latter group developed pendulous crops while under observation in the laboratory at Galveston, Texas.

The diet fed and the age when pendulous crop first appeared are not known, except in 4 of the turkeys which were fed commercial chicken ration supplemented with cracked corn. The pendulous crop was obvious in these turkeys at 79, 88, 138, and 365 days of age.

Immediately after the birds were killed by severing the cervical portion of the spinal cord, the feathers were removed and the skin was dissected from the cervical region. In some cases, the esophagus and crop were filled with a 4.0 per cent solution of formaldehyde to obtain better preservation of the tissue. Multiple sections of the wall of the crop and striated muscle from the thorax and extremities were obtained for histologic study. All tissues were fixed in a 4.0 per cent solution of formaldehyde, prepared by the paraffin technique, and stained routinely with hematoxylin and eosin. Selected sections were

Fig. 1—Attachment of suspensory muscle to crest of sternum (A) and fibrous tissue between suspensory muscle and pectoral muscle (B). Fibrous tissue on right has been removed to show underlying crop (C).

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* Cerelose, produced by Corn Products Refining Co., Argo, Ill.

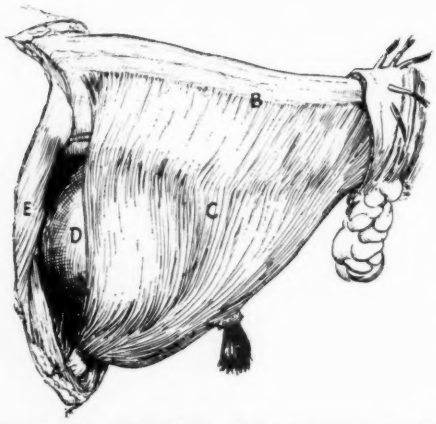

Fig. 2—Line of attachment of the suspensory muscle in a tendinous-like band along the lateral side of the neck. Tendinous attachment (A, B); suspensory muscle (C); crop (D); and pectoral muscle (E).

stained by the van Geison-Verhoff technique. Gomori's methenamine silver and periodic acid-Schiff stains were used for glycogen detection.

OBSERVATIONS

In the normal turkey, there is a muscle attached by a thin tendinous band to the anterior surface of the crest of the sternum (fig. 1). The muscle continues anteriorly as a wide, thin muscle and is inserted into the skin of the anterior portion of the neck and along a tendinous attachment that passes superficially to the longus colli group of muscles on the lateral side of the neck (fig. 2). There is a narrow area of dense fibrous tissue between the lateral margins of this muscle and the anterior border of the pectoralis muscles (fig. 3). There are varying amounts of fat and fibrous tissue between the crop and the inner surface of this suspensory muscle, and the crop can be easily dissected away.

In the younger birds and the older female turkeys, there was little, if any, fat in the subcutaneous tissues overlying this muscle. In the older male turkeys, there was a thick layer of subcutaneous fat. The thickness of the muscle varied in different birds. In some of the turkeys, this muscle formed a continuous covering over the crop, while in normal birds there were spaces of varying size between the muscle bundles (fig.4).

The muscle in the anterior cervical region of the turkey with pendulous crop resembled that in the normal bird. Focal areas of hemorrhage were present in the muscles of some birds. There were variations in the thickness of the suspensory

Fig. 3—There is a narrow area of fibrous tissue between the lateral margin of the suspensory muscle and the anterior border of the pectoralis major muscles. Muscle has been cut and reflected. Suspensory muscle (A); crop (B); fibrous tissue (C).

Fig. 4—Drawing showing spaces between muscle bundles in the crop of a normal turkey.

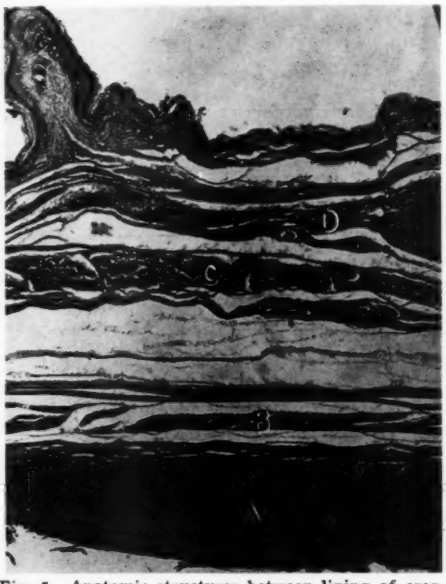

Fig. 5—Anatomic structures between lining of crop and skin. Squamous epithelium lining crop (A); smooth muscle in wall of crop (B); suspensory muscle (C); dermal muscle (D); and skin (E). H & E stain; x 31.

Fig. 6—Pendulous crop.

muscle in the different turkeys with pendulous crops similar to that observed in the turkeys with normal crops. The fibrous tissue at the lateral margin of the suspensory muscle was stretched in the birds with very large crops. As one would expect, varying amounts of food and liquid were present in the pendulous crops. However, nothing was observed within the crop to suggest mechanical obstruction. The wall and mucosa of the crop usually were grossly normal. In 1 of the birds, there was a localized, ulcerated area in the skin and subcutaneous tissue on the inferior surface of the enlarged crop. In some turkeys with pendulous crops, the mucosa of the crop had focal areas of ulceration.

The structures within the wall of the crop, from the squamous epithelium outward to the skin, are shown (fig. 5). These included squamous epithelium, smooth muscle, and a zone of fibrous tissue which separated the muscle layers of the crop from the striated suspensory muscle. A zone of fibrous tissue, containing varying amounts of fat and small dermal muscles, lay between the suspensory muscle and the skin. The gross appearance of a bird with pendulous crop is shown (fig. 6).

Histologically, the thickness of the suspensory muscle varied, and sometimes it was completely absent in focal areas in both the normal turkeys and those with pendulous crops. Some of the birds with pendulous crops had an acute and chronic inflammatory reaction within the wall; however, this was not a characteristic finding in all birds. The degree of involvement of the muscle and also the age of the

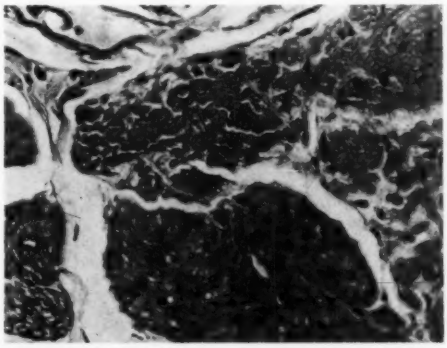

Fig. 7—Focal areas of degenerated muscle in wall of crop (A) stain red, while adjacent normal muscle (B) stains pink. H & E stain; x 326.

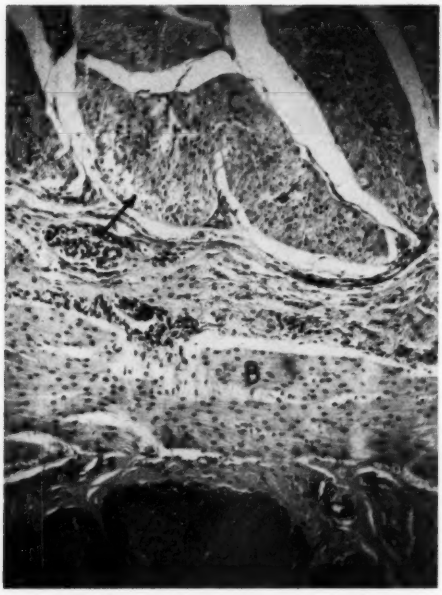

Fig. 8—There are focal areas of vacuolation in smooth muscle of crop (arrow). Squamous epithelium lining crop (A); inner layer of muscle (B); outer layer of muscle (C). H & E stain; x 88.

degenerative change varied within the same bird. In the smooth muscle of the wall of the crop, there were focal areas that stained red, in contrast to the pink-staining homogeneous normal muscle. Similar changes were present in the small muscles associated with the feather follicles (fig. 7). Often there were focal, light-staining, vacuolated areas in the muscle bundles in the wall of the esophagus (fig. 8, 9) and in the wall of the crop. In some turkeys, the striated muscle in the wall of the crop was completely absent in focal areas (fig. 10). In such areas, fibrous tissue appeared to replace the degenerated muscle. Degenerative changes in the muscles of the turkeys with pendulous crops were not limited to the crop muscle. The birds with the lesions in the esophagus (fig. 8, 9) also had an acute degenerative change in the right pectoral muscle (fig. 11). Turkeys with pendulous crops usually had degenerative changes in muscles other than those in the wall of the crop.

Degenerative changes, similar to those in birds with pendulous crops, were present in the muscles of apparently normal turkeys. Turkey X2195 was an adult, normal

Fig. 9—Vacuoles in muscle seen in figure 8. H & E stain; x 418.

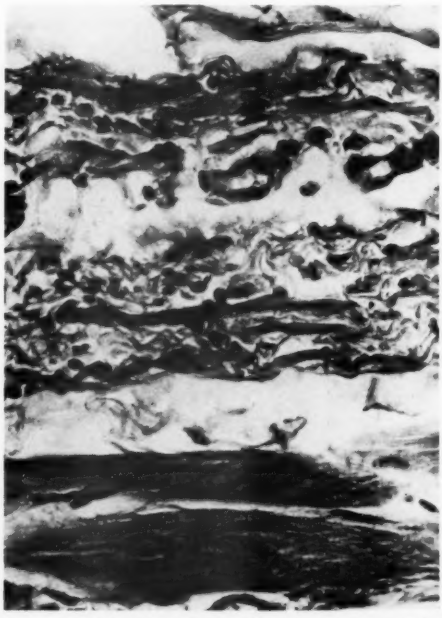

Fig. 10—Extensive degeneration has occurred in striated muscle in wall of crop. H & E stain; x 456.

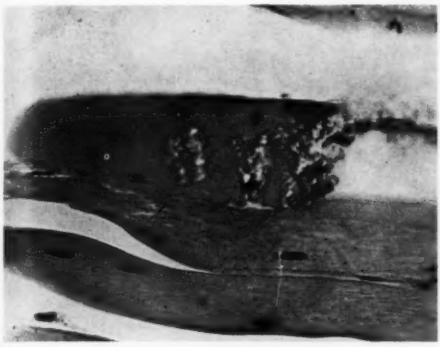

Fig. 11—Acute degenerative changes are present in pectoral muscle. Degenerative changes also are present in wall of crop of same bird (see fig. 8, 9). H & E stain; x 350.

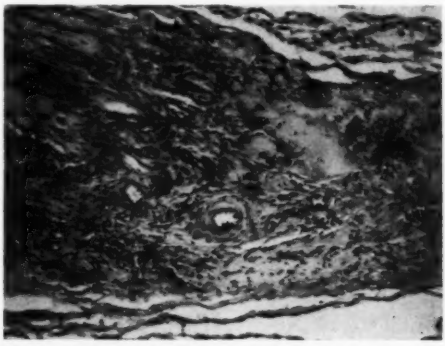

Fig. 12—Crop is grossly normal. Extensive degeneration of striated muscle of crop apparently has occurred, since area is fibrotic. H & E stain; x 75.

female, weighing 14 lb. Changes present in the small muscles in the skin were similar to those previously described in birds with pendulous crop. Extensive degeneration also was present in a local area of striated muscle in the wall of this crop (fig. 12). Considerable fibrosis was present. This lesion is interesting, since there were leukocytes and monocytic cells infiltrating the area. A more acute degenerative change was present in the striated muscle of the crop in turkey 256. This turkey was hatched and kept in our laboratory until he was killed when 97 days old. At no time did the crop of this turkey appear enlarged. The necrosis of the crop muscle is shown (fig. 13).

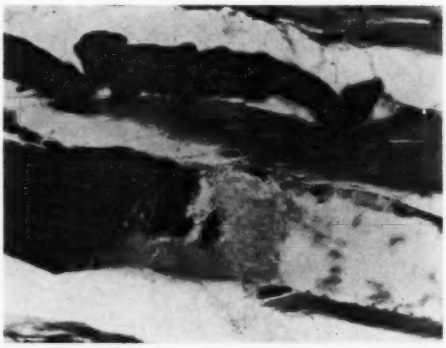

Fig. 13—Acute degeneration in striated muscle of crop on 97th day. No pendulous crop. H & E stain; x 350.

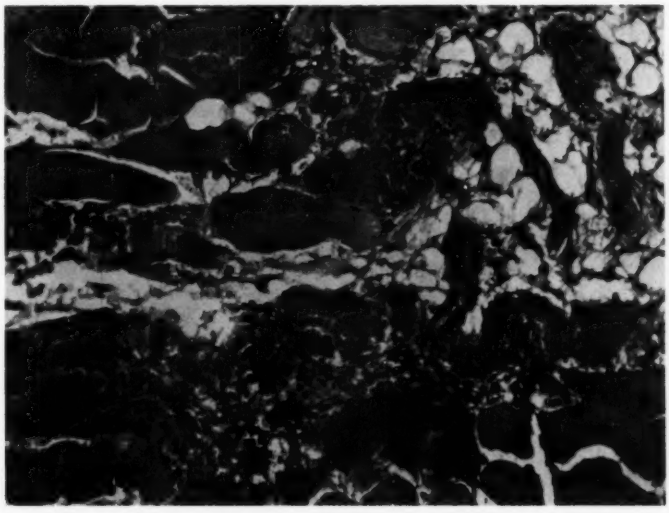

Fig. 14—Focal area of necrosis in muscle from thigh. Associated with some of focal areas of necrosis are mononuclear cells. H & E stain; x 133.

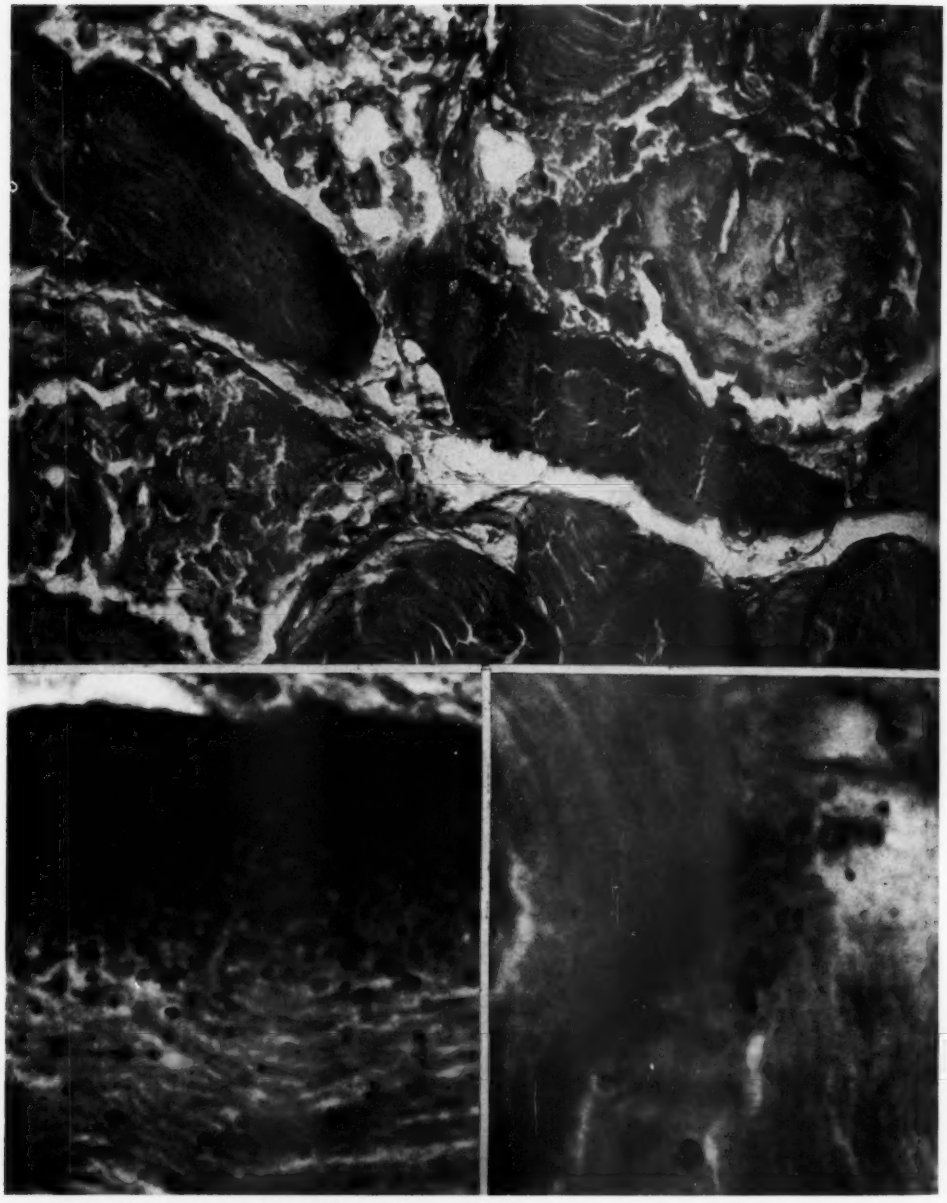

Fig. 15A—Glycogen-staining bodies (black) in normal-appearing muscle about an area of necrosis. Gomori's methenamine silver; x 270.

Fig. 15B—Higher magnification of these bodies. x 1,520.

Fig. 15C—Another area of muscle showing glycogen-staining bodies. x 1,790.

Degenerative changes in the muscles, other than those in the wall of the crop, have been a characteristic finding in turkeys from the same group as those with pendulous crops. Some of the latter birds had leg weakness, paralysis, and enlarged knee joints. The muscular degeneration in these birds was identical with that ob-

served in turkeys with and without pendulous crop. The occurrence of this muscular degeneration has not been restricted to any single group of turkeys that we have studied. One of the 4 turkeys obtained from Wisconsin did not have a pendulous crop but had extensive necrosis of the muscles removed from the legs, pectoral region, area of the cervical vertebrae, and within the pelvis (fig. 14). There was a variation in the degree of necrosis in the muscles from different areas of the body. Sometimes the necroses were associated with a mononuclear reaction or a proliferation of sarcolemmic nuclei.

With Schiff's periodic acid and Gomori's methenamine silver stains for glycogen, small round bodies in the muscle fibers were seen (fig. 15a–15c). These glycogen-staining bodies usually were approximately 1 μ in diameter; however, some appeared to be 2 to 3 μ. Usually, they were present at the margin of a muscle fiber (fig. 15a). The number present varied widely. Sometimes, the greater part of the muscle fiber in a local area was infiltrated. Eosin-staining granules in the routine hematoxylin-eosin preparation, similar in morphology to the glycogen-staining granules in a Schiff's periodic acid stain, were present in the areas of muscle necrosis but they did not stain positive for glycogen. Usually, the normal-appearing muscles adjacent to the necrotic foci had the largest number of these glycogen-staining bodies (fig. 15a). We have been unable to stain these granules with the usual bacterial and Giemsa stains. Further efforts are now being made to identify these granules.

The enlargement of the crop, once present, usually persisted. However, in 1 turkey, C76, the enlargement fluctuated during a period of a year. This turkey was 5 weeks of age when obtained from the Poultry Department of the A. & M. College of Texas. It was kept under close observation and fed a commercial ration prepared for chickens which was supplemented with cracked corn. When 8 months of age, the bird was returned to the Agricultural and Mechanical College of Texas for breeding. Upon its return to Galveston 4 months later, the crop was enlarged. However, after six days it was normal in size, but 2 months later was enlarged again. This enlargement of the crop and its spontaneous regression continued until the bird was killed at the age of 2 years. At the time of necropsy, the small blood vessels in the skin at the inferior margin of the crop were dilated. The crop contained a moderate amount of dry grass, particles of the commerical food, and a moderate amount of liquid. The mucosa was grossly normal. Histologic studies were not made from the tissues of turkey C76.

Discussion

There was no anatomic difference in the musculature of the cervical region in turkeys with or without pendulous crops. Degenerative changes were usually present in the muscles of the wall of the crop and in the suspensory muscle that normally supports the crop. Similar histologic degenerative changes were present in the muscle of the pectoral region and from the extremities of some of the turkeys with pendulous crops. Degenerative changes in muscles other than those in the cervical region would suggest the mechanism for the development of pendulous crop. The musculature supporting the crop degenerates and the remaining muscle fibers stretch, resulting in a markedly distended crop. The muscle supporting the crop would be one of the first to show the clinical effect of this degenerative process, since it normally is stretched after the intake of large amounts of food. With the loss of elasticity, the crop would remain distended.

It is of considerable interest to find similar histologic changes in the muscles of "normal turkeys" and birds with muscular weakness that have been kept under the same environmental conditions as those with enlarged crops. This muscular degeneration has been observed in turkeys obtained from Texas, California, Minnesota, and Wisconsin. Obviously it is not a local lesion. Chickens in contact with the turkeys in our laboratory have a similar degeneration of their muscles. Similar glycogen-staining bodies are present in the chicken as well as in the turkey. Since the findings are suggestive of an infection, attempts are being made to isolate the agent. A nutritional deficiency also is being considered.

Summary

The muscle in the anterior cervical region that supports the crop of the turkey is a thin, striated muscle attached to the anterior crest of the sternum. It is inserted

into the skin on the anterior surface of the neck and into a tendinous attachment that passes superficially to the longus colli group of muscles and is attached on the lateral side of the neck.

No gross anatomic differences were present in muscles of normal birds and those with pendulous crops. Histologically, degenerative changes were present in the muscles of the wall of the pendulous crop. Similar changes also were present in muscles elsewhere in the body.

Like degenerative changes were present in the muscles of turkeys that clinically had normal crops but were kept under the same experimental conditions as birds that developed pendulous crop. This would suggest that the occurrence of a large crop indicates the degree and anatomic site of muscular degeneration.

Observations made during this study suggest an infectious agent or agents as the cause of the muscular degeneration. Attempts are being made to isolate the agent or agents, as well as considering a vitamin deficiency.

References

[1] Asmundson, V. S., and Hinshaw, W. R.: On the Inheritance of Pendulous Crop in Turkeys (*Meleagris galloparo*). Poult. Sci., 17, (1938): 276.

[2] Cline, L. E.: Turkey Production. Judd, Orange Publishing Co., New York City, (1933): 436.

[3] Hinshaw, W. R., and Asmundson, V. S.: Observations on Pendulous Crop in Turkeys. J.A.V.M.A., 88, (Feb., 1936): 154.

[4] Manfre, A. S., Wheeler, H. O., Feldman, G. L., Rigdon, R. H., Ferguson, T. M., and Couch, J. R.: Fungi in the Crop of the Turkey. Am. J. Vet. Res., 19, (July, 1958): 689–695.

[5] Rigdon, R. H., Ferguson, T. M., Feldman, G. L., Wheeler, H. O., and Couch, J. R.: A Study of the Mechanism of the Experimentally Induced Pendulous Crop in Turkey. Am. J. Vet. Res., 19, (July, 1958): 681–688.

[6] Wheeler, H. O., Crawford, W. P., and Couch, J. R.: Pendulous Crop in Turkeys. Poult. Sci., 36, (1957): 1167.

SUMMARIO IN INTERLINGUA

Ingluvies Pendule in Gallos de India: Un Studio Anatomic e Pathologic

Le musculo in le region antero-cervical que supporta le ingluvie del gallo de India es tenue e striate e es attachate al crista anterior del sterno. Illo es inserite in le pelle del facie anterior del collo e in un attachamento tendinose que passa superficialmente al gruppo de musculos longe del collo e es attachate al latere del collo.

Nulle differentias macro-anatomic esseva notate in ille musculos inter aves normal e aves con ingluvie pendule. Histologicamente, alterationes degeneratori esseva presente in le parietes muscular del ingluvie pendule. Simile alterationes esseva etiam presente in le musculos in altere partes del corpore.

Le mesme typo de alterationes degeneratori esseva presente in le musculos de gallos de India que habeva clinicamente normal ingluvies sed que habeva vivite sub le mesme conditiones experimental como le aves que disveloppava ingluvies pendule. Isto suggere que le occurrentia de un grande ingluvie indica le grado e le sito anatomic de degeneration muscular.

Observationes facite in le curso del presente studio suggere le existentia de un agente infectiose o de plure tal agentes como le causa del degeneration muscular. Es in progresso effortios a isolar le agente o le agentes in question. Le possibile existentia de un carentia de vitamina es etiam sub consideration.

Studies of Newcastle Disease Virus Strains in Various Cell Cultures

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NEWCASTLE DISEASE VIRUS (NDV) has been propagated in different tissue culture systems. Scott *et al.*[7] described the method of culturing NDV in isolated chorioallantoic membranes suspended in Tyrode's solution. Growth of NDV was reported in HeLa cells by Tyrell,[9] in monkey kidney cell culture by Chanock,[2] and in human embryonic lung cell culture by Chaproniere and Pereira.[3] Kohn and Goldwasser[4] described the multiplication of a cytopathogenic strain of NDV in monolayers of chicken embryo cells. Shimizu *et al.*[8] successfully propagated different strains of NDV in swine kidney cell culture. Bankowski[1] developed a vaccine which was prepared with a live, avirulent strain of NDV modified by passage in minced chicken embryo cell culture. Some chemical factors influencing growth of NDV in isolated chorioallantoic membranes were reported by Zuschek *et al.*[11]

The investigation reported here concerns the growth and the behavior of several strains of NDV in different cell culture systems, with particular reference to immunologic changes. Since cells of chicken embryo origin may introduce other viruses that occur in chickens, special emphasis was given to the study of NDV strains in cell cultures of mammalian origin.

MATERIALS AND METHODS

Titrations of virus were done in developing chicken embryos and in roller tube cultures of the various cells. The immunogenic potencies of viruses grown in cell culture were tested in chicks of different ages.

Virus Strains.—The four strains* of NDV used in this study were: the B1 strain, which is an attenuated virus isolated by S. B. Hitchner and generally is used for the vaccination of 1-day-old chicks; the Roakin strain, which was isolated from 1-month-old chicks by F. R. Beaudette and which produces a mild systemic reaction, sneezing, and less than 0.5 per cent mortality; the Mass.—MK107—1949 strain, which was isolated by H. van Roekel and attenuated by cultivation in duck embryos; and the Texas-GB-1948 strain, which was isolated by W. A. Boney from an epornitic of severe nervous-respiratory infection of chickens and has since been maintained as a virulent, highly antigenic virus. Stock virus for each strain for these experiments consisted of infected chicken embryo allantoic fluid, maintained at −80 C.

Cell Cultures.—All primary monolayer cell cultures (chicken embryo, embryonic calf kidney, dog kidney) were prepared by a modification of the method of Youngner.[10] Continuous cell lines (HeLa, Hep-2, human heart) were harvested by trypsinization and washed with growth medium before being transferred to bottles or tubes of the various growth mediums. Both the primary and continuous cell lines were propagated in 32 oz. vaccine bottles or roller tubes, depending on the intended use (table 1). Penicillin (200 units per ml.) and streptomycin (200 μg. per ml.) were added routinely to all cell cultures; pH was adjusted with sodium bicarbonate. Before inoculation of cultures with virus, growth mediums were replaced with synthetic medium 199[5] without serum.

Chicken Embryo Infectivity Test.—Viruses propagated in cell cultures were titrated by inoculating 0.1 ml. of tenfold dilutions of the virus suspensions into 9- to 10-day embryos which then were incubated at 36 C. for two to four days and killed by chilling. The infective titer was determined by the death ratio of the chicken embryos and by the hemagglutination (HA) activity of the allantoic fluids. Three eggs were used per dilution and the 50 per cent infective dose ($i.d._{50}$) was calculated by the method of Reed and Muench.[6] In the HA tests, 0.5 per cent chicken red blood cell suspensions were mixed in equal volumes (0.5 ml.) with serial twofold dilutions of the material to be tested for HA activity, and allowed to settle for 30 minutes at room temperature. The virus-containing cell culture fluids could not be used for a direct HA test because they had no HA activity detectable by the usual techniques. The reason for the lack of HA activity in the cell culture fluids was not investigated.

Virus Titrations in Cell Culture Tubes.—Serial tenfold dilutions of the cell-cultured strains were inoculated into roller tubes containing the same type cells as those in which the virus was initially propagated. The cytopathic effects of the virus strains were degeneration of the cytoplasm with destruction of the cell layer. The majority of degenerated cells fell away from the wall of the roller tubes. These cytologic changes occurred

From the Biological Laboratories, Pitman-Moore Co., Indianapolis, Ind.

* Obtained from the Department of Veterinary Science, University of Wisconsin, Madison.

TABLE 1—Cell Culture Growth Mediums Used for Propagating Newcastle Disease Virus

Tissue cells	Cell counts in 1 ml.	Basal solution	Lactalbumin hydrolysate	Serum
Human heart	150,000	Eagle's	.....	Calf (10%)
HeLa	125,000	Eagle's	.....	Horse (10%)
Hep-2	125,000	Eagle's	.....	Calf (20%)
Chicken embryo	300,000	Earle's BSS	0.25%	Calf (5%)
Dog kidney	75,000	Earle's BSS	0.25%	Horse (10%)
Embryonic calf kidney	150,000	Earle's BSS	0.25%	Horse (10%)

two to four days after virus inoculation, the time interval depending on the concentration of virus inoculated.

Potency Tests in Chicks.—The antigenic potency of the cell culture propagated strains was tested by vaccinating chicks and subsequently exposing them to virulent virus. The B1 strain was tested in 1- to 5-day-old chicks; MK107, Roakin, and killed GB strains were tested in 3- to 5-week old chicks. The B1 strain was inoculated ocularly or intranasally; MK107 and Roakin strains were inoculated by the wing-web method; the killed GB-Texas strain (0.5 ml.) was inoculated intramuscularly. In the ocular or intranasal method, a special applicator was used to place 1 drop of the virus suspension on the open eye or into the nostril. In the wing-web method, the inoculating needle first was dipped into the virus suspension, then was pushed from under the wing completely through the web with care to avoid the muscles and bones. Two weeks later, the vaccinated chicks and 10 or more control chicks were exposed to at least 10,000 lethal doses of virulent GB-Texas virus, and then observed for two weeks. Those with incoordination, tremors, paralysis, or death were recorded as infected. The controls all developed typical signs or died within the two-week observation period.

The results of the different titration methods were identical in most of the cases. In some cases, however, the titrations in cell culture tubes resulted in higher titers than in the chicken embryo infectivity tests.

RESULTS

The virulence or infectivity of the stock virus strains ranged within definite limits (table 2).

Generally, cell cultures were seeded with 10^3 i.d.$_{50}$ of virus. The incubation period of the strains in the cells at the temperature used (37 C.) was about three days. However, GB-Texas and B1 developed somewhat faster and were ready for harvest after two days (table 3).

Ten to 20 serial passages of the different virus strains were made in cell cultures. In most cases, the virus strains were readily adapted to the different tissues, developing high titers in the first passages and only a slight rise in subsequent passages. However, in some cases, the titer of the strains did increase 2 to 3 logs in the course of the ten to 20 passages, as was observed with the B1 strain in human heart and Hep-2 cells, and with the Roakin strain in the HeLa, Hep-2, and calf kidney cells.

TABLE 2—Titers of Stock Viruses (Chicken Embryo Allantoic Fluid) Used in Studies of Newcastle Disease Virus Strains

NDV strain	Log of i.d.$_{50}$/ml.
GB-Texas	9.0–10.0
Roakin	9.0–10.0
MK107	9.0–10.5
B1	8.0–10.0

Since the virus strains used in these experiments had different characteristics, they will be discussed separately to point out their behavior in the cell cultures with special reference to their growth, virulence, and antigenicity.

B1 Strain.—For the study of the B1 strain, HeLa, Hep-2, and human heart cells were used. The summarized figures (table 4) represent the average titers of the higher passages.

It is interesting that the B1 strain in Hep-2 cells appeared only in the first passage, in which it showed its original seed titer. No evidence was observed that virus was present in subsequent passages. However, there was definite propagation of the B1 strain in human heart cells, but five passages were required to reach a high and apparently maximum titer (table 5). Simi-

TABLE 3—The Influence of the Time of Harvest on the Titers of Strains of Newcastle Disease Virus in Human Heart Cells Determined by Chicken Embryo Infectivity Tests

	Log of i.d.$_{50}$/ml. after indicated hours			
NDV strain	24	48	72	96
B1	4.0	7.5	7.5	6.5
Roakin	4.0	5.0	7.5	7.5
MK107	3.0	4.0	6.5	6.5
GB-Texas	4.0	6.0	6.5	6.0

TABLE 4—Titer and Potency of the B1 Strain of Newcastle Disease Virus

Cell cultures	Titers based on			Potency in chicks	
	CPE in homologous cells	Lethal effect in chicken embryos	Egg infectivity titrated by HA activity of allantoic fluid	immune/ inoculated	immunized (%)
HeLa	6.0	7.0	7.0	0/20	0
Hep-2	NT	NT	NT	.....	..
Human heart	8.0	7.5	7.5	7/40	17.5

CPE = cytopathic effect. NT = no virus titer evident.

lar results were obtained with passages of the B1 strain in HeLa cells.

The immunogenic effect of the cell-cultured B1 strain was investigated by vaccinating 1-day-old chicks ocularly. In repeated experiments, the vaccinated chicks failed to develop an immunity to challenge with the virulent virus strain (table 4). At the same time, 1-day-old chicks inoculated with chicken embryo vaccine developed good immunity.

TABLE 5—Titers of the B1 Strain of Newcastle Disease Virus at Successive Passages in Human Heart Cells

Passage No.	CPE in homologous cells	Lethal effect in chicken embryos	Egg infectivity determined by HA activity of allantoic fluid
1	NT	4.0	5.0
2	4.0	5.0	5.0
3	NT	6.0	6.0
4	....	5.0	5.0
5	5.0	8.0	8.0
6	....	7.0	7.0
7	7.0	4.0	7.0
8	....	7.0	7.0
9	8.0	8.0	8.0
10	6.0	8.0	7.0
11	8.0	7.0	7.0
12	8.0	9.0	8.0
13	8.0	7.0	7.0
15	5.0	7.0	7.0
16	8.0	7.0	7.0
18	8.0	7.0	7.0

CPE = cytopathic effect. NT = no virus titer evident.

MK107 Strain.—The MK107 strain also was studied in HeLa, Hep-2, and human heart cells (table 6). The titers indicated satisfactory growth of MK107 in all of these cells, good growth being evident in the first passages. In 20 subsequent passages, the titer increased no more than 1 log.

The cell-cultured MK107 strain also was used to vaccinate 3- to 5-week-old chicks by the wing-web method. The high level of protection against virulent virus indicated a good antigenicity, but relative virulence for the 3-week-old chicks, which did not decrease with continued cell passages, proved to be a serious disadvantage. In fact, the virulence of the passaged virus was sometimes higher for 3-week-old chicks than that of a comparable amount of the original chicken embryo propagated virus. In some cases, the cell-cultured virus paralyzed or killed a considerable number (10 to 50%) of the vaccinated chicks.

Roakin Strain.—Propagation of this strain was attempted in HeLa, Hep-2, human heart, chicken embryo, embryonic calf kidney, and dog kidney cells (table 7). Ten passages were made in all cells except the dog kidney cells, in which no further attempts were made after five blind passages which gave no evidence of virus propagation.

Titers of the Roakin strain in chicken embryo, HeLa, Hep-2, human heart, and embryonic calf kidney cells were high, and gradually increased by 2 to 3 logs during passage.

Vaccination of 3- to 5-week-old chicks by the wing-web method with cell-cultured

TABLE 6—Titer and Potency of the MK107 Strain of Newcastle Disease Virus

Cell cultures	Titers based on			Potency in chicks	
	CPE in homologous cells	Lethal effect in chicken embryos	Egg infectivity titrated by HA activity of allantoic fluid	immune/ inoculated	immunized (%)
HeLa	7.0	7.5	7.5	.....	....
Hep-2	8.0	6.5	6.5	62/66	94
Human heart	8.0	7.0	7.0	69/71	97

CPE = cytopathic effect.

Roakin strain resulted in excellent immunity. It is evident that this virus can be propagated in several types of cell culture without increasing in virulence for chicks and without losing its antigenic properties.

GB-Texas Strain.—This strain grew readily in human heart, HeLa, Hep-2, and chicken embryo cells and reached a high titer by the second day of incubation (table 8). The titer was high in all of the early passages and did not change significantly in the subsequent passages.

With the exception of the HeLa cell cultures, GB-Texas strain propagated in cell cultures was inactivated with a sufficient amount of a 10 per cent solution of *beta*-propiolactone to yield a final concentration of 0.1 per cent. Aluminum hydroxide solution (1.2 mg. per ml.) was also added at the ratio of 40 ml. to 100 ml. of virus material. The immunity of 3-week-old chicks, vaccinated intramuscularly with a single 0.5 ml. dose of this inactivated and adsorbed virus, was challenged two weeks later with 0.2 ml. of virulent virus containing at least 10,000 $l.d._{50}$. The vaccine prepared from the chicken embryo cell virus gave good immunity, but the virus propagated in Hep-2 and human heart cells was found to have a marked loss in antigenicity.

The virus propagated readily in suspended cell cultures of minced chicken embryo tissues in a glass fermentator. Peak titer occurred as early as 24 hours after inoculation. Further studies are in progress for the evaluation of the virus propagated by this method.

TABLE 7—Titer and Potency of the Roakin Strain of Newcastle Disease Virus

Cell cultures	Titers based on: CPE in homologous cells	Titers based on: Lethal effect in chicken embryos	Titers based on: Egg infectivity titrated by HA activity of allantoic fluid	Potency in chicks: immune/inoculated	Potency in chicks: immunized (%)
HeLa	8.0	8.0	8.0	20/20	100
Hep-2	9.0	8.5	8.0	19/19	100
Human heart	8.0	7.5	7.5	19/19	100
Chicken embryo	8.0	8.0	8.0	.....	.....
Embryo calf kidney	8.0	7.0	7.0	17/20	85
Dog kidney	NT	NT	NT	.....	.....

CPE = cytopathic effect. NT = no virus titer evident.

DISCUSSION

The purpose of these investigations was to study the propagation of various NDV strains in different cell cultures and to study vaccines prepared from cell-cultured virus strains. To be satisfactory for use as a live-type vaccine, a tissue culture virus strain must meet three requirements: (1) attainment of satisfactory titer, (2) modification of virulence, and (3) maintenence of antigenicity.

TABLE 8—Titer and Potency of the GB-Texas Strain of Newcastle Disease Virus

Cell cultures	Titers based on: CPE in homologous cells	Titers based on: Lethal effect in chicken embryos	Titers based on: Egg infectivity titrated by HA activity of allantoic fluid	Potency in chicks: immune/inoculated	Potency in chicks: immunized (%)
HeLa	8.0	7.0	7.0	.....	....
Hep-2	8.0	7.0	7.0	7/35	20
Human heart	8.0	7.0	7.0	1/35	3
Chicken embryo	8.0	7.5	7.5	17/19	89.5

CPE = cytopathic effect.

All the strains used in these experiments were found to propagate satisfactorily in HeLa and human heart cells. Hep-2 cells were excellent for the growth of GB-Texas, MK107, and Roakin strains, but did not support the propagation of the B1 strain. In the cell cultures in which it grew, the B1 strain lost its antigenicity, and therefore is not satisfactory as a vaccine.

The MK107 strain also was unsatisfactory as a vaccine since, while it retained

its high antigenicity when propagated in the cell cultures, it remained virulent. Only the Roakin strain satisfied all three requirements. It grew well in all cultures except the dog kidney cell, it did not cause any reactions or signs in the inoculated animals, and it gave a high level of protection.

For preparation of a killed virus vaccine, virulence is not especially important, so long as antigenicity is maintained at a high level. Hence, it is possible to use a highly virulent and antigenic virus such as the GB-Texas strain, which grew very well in the various cell cultures, especially in chicken embryo cell culture. The only question remaining concerned antigenicity of the killed cell-cultured virus vaccines. It was interesting to learn that vaccine produced from the virus grown in human heart and Hep-2 cells resulted in a relatively poor immune response; vaccine prepared from chicken embryo cell culture virus possessed good antigenicity (table 7). This finding promises a new approach for the preparation of killed NDV vaccines.

SUMMARY

Evidence is presented regarding the suitability of several Newcastle disease virus (NDV) strains propagated in various tissue culture systems for vaccination purposes. The various strains differed considerably in essential characteristics.

The B1 strain grew well in HeLa and human heart cells, but the cell-propagated virus lost its antigenicity and failed to stimulate immunity in the vaccinated chicks.

The MK107 strain grew well in HeLa, Hep-2, and human heart cells. This strain did not lose its antigenicity during the cell culture passages, but the relative virulence of the strain limited its use for vaccination purposes.

Roakin strain was successfully propagated in HeLa, Hep-2, human heart, chicken embryo, and embryonic calf kidney cells. The cell-cultured virus stimulated the production of good immunity in vaccinated chicks.

The GB-Texas strain grew readily in human heart, HeLa, Hep-2, and chicken embryo cells. This strain was highly virulent but, when inactivated with *beta*-propiolactone and adsorbed to aluminum hydroxide, the strain grown in chicken embryo cells was suitable for use as an inactivated vaccine. However, when propagated in Hep-2 and human heart cells it was significantly reduced in antigenicity.

References

[1] Bankowski, R. A.: A Modified Live Newcastle Disease Virus Vaccine. Proc. Soc. Exptl. Biol. & Med., 96, (1957): 114–118.

[2] Chanock, R. M.: Cytopathogenic Effect of Newcastle Disease Virus in Monkey Kidney Cultures and Interference with Poliomyelitis Viruses. Proc. Soc. Exptl. Biol. & Med., 89, (1955): 379–381.

[3] Chaproniere, D. A., and Pereira, H. G.: Propagation of Fowl Plague and Newcastle Disease Viruses in Cultures of Embryonic Human Lung. Brit. J. Exptl. Pathol., 36, (1955): 607–610.

[4] Kohn, A., and Goldwasser, R.: Multiplication of Newcastle Disease Virus in Chick Embryo Tissue Culture. Proc. Soc. Exptl. Biol. & Med., 96, (1957): 198–200.

[5] Morgan, J. F., Morton, H. J., and Parker, R. C.: Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. Proc. Soc. Exptl. Biol. & Med., 73, (1950): 1–8.

[6] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

[7] Scott, G. R., Hanson, R. P., and Brandly, C. A.: A Simplified Procedure for the *in Vitro* Propagation of Newcastle Disease Virus. Proc. Book, AVMA, (1954): 312–315.

[8] Shimizu, T., Ishizaki, R., Kono, Y., Isshii, S., and Matumoto, M.: Multiplication of Newcastle Disease and Fowl Plague Viruses in Swine Kidney Tissue Culture. Bull. Nat. Inst. Anim. Health, Tokyo, Japan, 33, (1957): 49–59.

[9] Tyrell, D. A. J.: New Tissue Culture Systems for Influenza, Newcastle Disease and Vaccinia Viruses. J. Immunol., 74, (1955): 293–305.

[10] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., 85, (1954): 202–205.

[11] Zuschek, F., Hanson, R. P., and Brandly, C. A.: Factors Influencing the Growth of Newcastle Disease Virus in Isolated Chorioallantoic Membranes. Am. J. Vet. Res., 19, (Jan., 1958): 191–195.

SUMMARIO IN INTERLINGUA

Studios con Racias del Virus de Morbo de Newcastle in Varie Histoculturas

Es presentate observationes relative al qualification, pro objectivos de vaccination, de plure racias de virus de morbo de Newcastle propagate in varie systemas de histoculturation. Le racias usate differeva considerabilemente in characteristicas essential.

Le racia B1 ereseeva ben in cellulas HeLa e in cellulas de corde human, sed le cytopropagate virus perdeva su antigenicitate e non stimulava immunitate in vaccinate gallinettas.

Le racia MK107 ereseeva ben in cellulas HeLa, cellulas Hep-2, e cellulas de corde human. Iste racia non perdeva su antigenicitate durante le passages cytocultural, sed le relative virulentia de illo restringeva su utilisabilitate pro objectivos de vaccination.

Le racia Roakin esseva propagate a bon successo in cellulas HeLa, Hep-2, de corde human, de embryones de gallina, e reno-embryonic de vitello. Le cytoculturate virus stimulava le production de bon immunitate in vaccinate gallinettas.

Le racia GB Texas ereseeva ben in cellulas de corde human, HeLa, Hep-2, e embryonic de gallinetta. Iste racia esseva fortemente virulente, sed quando illo esseva inactivate con *beta*-propiolactona e adsorbite a hydroxydo de aluminium, le racia GB-Texas passate per cellulas embryonic de gallina esseva utilisabile como vaccino inactivate. Tamen, quando le terreno de culturation esseva cellulas Hep-2 o cellulas de corde human, le antigenicitate de iste racia esseva significativemente reducite.

The Trigeminal and Facial Nerves as Pathways for Infection of Sheep with *Listeria monocytogenes*

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LISTERIOSIS in sheep may occur as a localized infection of the central nervous system. It may also occur as a generalized disease with focal necrosis in the liver or heart. In the latter form, Listeria organisms can be found both in the central nervous system and the visceral organs.[22] The generalized infection may also be associated with the death of fetuses and with subsequent abortion.[24] The infection and lesions of the encephalitic form seem to be confined largely to the brain and spinal cord, producing signs of paresis or flaccid paralysis.[22]

The encephalitic form of listeriosis has been irregularly produced in sheep experimentally given *Listeria monocytogenes* alone.[7, 11, 14, 19, 21]

In an attempt to explain the pathogenesis of listeriosis, Pallaske [23] suggested that, after primary infection in the nasal cavity, the route to the brain was by way of the lymph vessels of the olfactory nerve to the spaces of the subarachnoid area which, in turn, led to infection of the perivascular spaces in the brain substance.

Asahi *et al.*[1-6] investigated the mechanism by which the encephalitic disease occurred. *Listeria monocytogenes* was inoculated into the lip by making minute wounds with a hypodermic needle or by feeding beans (sharp edges lacterated the oral mucosa) seeded with Listeria organisms. These experiments resulted in encephalitic listeriosis in 6 of 18 sheep. Clinical findings and histologic lesions of the trigeminal nerves in these animals were similar to those observed in naturally infected animals. *Listeria monocytogenes* was isolated from the site of the lesions. These authors also found cellular reactions at the root of either one or both trigeminal nerves of naturally infected sheep and goats.[4, 6] From these observations, they favored the hypothesis that the route of natural infection followed the trigeminal nerves to the brain stem.

Cordy and Osebold,[8] as a result of their histologic studies of 15 sheep, disagreed with the idea of centripetal invasion along the nerves and supported the concept of a hematogenous route of infection.

This is a report of findings in 17 sheep, in which *L. monocytogenes* was inoculated directly into the branches of the trigeminal or facial nerves. The intraneural exposure was designed to test the idea that these cranial nerves may be a pathway for infection of the central nervous system by *L. monocytogenes*.

MATERIALS AND METHODS

Strain 1501-2 of *L. monocytogenes* was obtained from the medulla of a sheep which had died from listeriosis in 1951. The culture had been kept in a lyophilized state since that time. This culture, after reconstitution, was used to expose 10 sheep (15, 23, 33, 34, 56, 97, 859, 865, 906, 912). The second strain (12255), obtained from M. L. Gray of Bozeman, Mont., was used alone to expose 3 sheep (676, 677, 678). It was isolated by Gray in 1951 from the medulla of a calf which had died of listeriosis and white muscle disease. It also was used in combination with strain 1501-2 to expose 2 other sheep (861, 864). A third source of organisms was the medulla oblongata of 1 of the experimental animals (864) infected with a mixture of the strains 1501-2 and 12255 in our trials. It was used to infect 2 sheep (243, 263).

The left infraorbital or superior buccal nerves of 17 sheep, 7 to 42 months old, were exposed aseptically under local anesthesia. Using a 27-gauge needle, the Listeria organisms were inoculated into the infraorbital nerve at its emergence from the infraorbital canal, or into the superior buccal nerve at a site ventral to the lateral commissure of the eye. The amount injected was 0.1 to 0.5 ml. of a broth suspension containing 10^6 to 10^9 organisms per milliliter. A dry surgical swab was applied to the site of inoculation to absorb inoculum that might leak outside the nerve bundle. In some sheep, sulfonamide-urea powder, quaternary ammonia solution in a 1:1,000 to 1:5,000 dilution, chlorhexidine diacetate, or nitrofurazone powder was applied to the surgical area.

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TABLE 1—The Appearance and Characteristics of Signs and Lesions in Sheep Given Listeria monocytogenes Intraneurally

	Clinical signs		Lesions	
Sheep No.	Days after inoculation	Characteristic	Days after inoculation	Characteristic
243	13–18	Paralysis, right side of face and tongue.	18	Meningo-encephalo-myelitis.
263	21–25 40–42 recovered	Ataxia; lameness right foreleg.	84	Not examined.
678	none	None.	116	Encephalitis.
861	33 recovered	Lameness, right foreleg.	94	Meningo-encephalo-myelitis.
864	27–37	Lameness, right foreleg.	37	Meningo-encephalo-myelitis.
912	17–22	Partial paralysis of nose, upper lip, and right ear; torticollis.	24	Meningo-encephalo-myelitis.
33	24–30	Paresis, forelegs; paralysis of left ear; torticollis.	33	Meningo-encephalo-myelitis.
677	none	None.	80	Meningo-encephalo-myelitis.
865	16–19 recovered	Paralysis of left ear and eye; hyperesthesia of face.	84	Meningitis-myelitis.

Sheep 33, 677, and 865 were inoculated via the left superior buccal nerve and the others were inoculated via the left infraorbital nerve.

The tissues taken for bacterial examination included portions of the olfactory lobe; parietal and occipital areas of the cerebrum; the cerebral peduncles; the pons; the corpus trapezoideum; the medulla; the cerebellum; the spinal cord near the medulla and at the lower cervical, upper thoracic, and the sacral levels; and the infraorbital, maxillary, trigeminal, superior buccal, and facial nerves. The suprapharyngeal, bronchial, mesenteric, and occasionally, presecapular lymph nodes were also examined. Samples of the heart, liver lungs, spleen, and kidneys were cultured for Listeria organisms.

The methods used for bacterial examination were essentially those described by Olson *et al.*[20] and Gray *et al.*[16]

Sections of tissue, adjacent to the sites from which bacteriologic specimens were taken, were fixed in 4 per cent formaldehyde for subsequnt histologic examination.

Paresis or flaccid paralysis of the head or limbs (with or without torticollis, which usually progresses to prostration and death) can be regarded as signs of the encephalitic form of listeriosis. Listeria organisms have been cultured from the central nervous system, and sometimes from the viscera as well,[22] in the encephalitic form of the disease. The microscopic lesions have been reported as meningo-encephalitis,[11] encephalitis,[9, 12] and encephalomyelitis.[8, 17]

The criteria for diagnosing the encephalitic form of listeriosis were (1) clinical signs, (2) histologic lesions similar to those of animals with naturally occurring cases, and (3) the distribution of *L. moncytogenes* as found in animals with naturally occurring cases.[22] Only when these three criteria were met, was an experimental animal considered to have encephalitic listeriosis.

RESULTS

Four (24%) of the 17 sheep inoculated with *L. monocytogenes* in the infraorbital (243, 864, 912) or superior buccal (33) nerves became moribund in 18 to 37 days and the course of disease met the criteria for encephalitic listeriosis.

In other sheep, some but not all of the criteria for encephalitic listeriosis were met.

Nine sheep had clinical signs or lesions of the central nervous system (table 1), but Listeria organisms were not recovered from 5 sheep. These 5 sheep were killed for examination 80 to 116 days after inoculation. Eight sheep had microscopic lesions of the central nervous system only. Lameness and subsequent flaccid paralysis of the forelegs occurred in 3 sheep (263, 864, 861). Paralysis, variable in extent, involved the lip, ear, eyes, nose, and tongue, in 4 (33, 243, 865, 912). These signs were confined to the right side in those sheep which had been inoculated in the left infraorbital nerve (243, 263, 861, 864, 912), and to the left side in (33, 865) sheep exposed in the left superior buccal nerve. Torticollis devel-

oped in 2 of the sheep (33, 912). The signs of disease first appeared 13 to 24 days after inoculation and persisted until 18 to 42 days after inoculation.

A febrile response, lasting 1 to 6 days after inoculation, occurred in all of the test animals. The onset of signs was usually correlated with a rise in body temperature (fig. 1, 2).

Four sheep which had encephalitic involvement clinically and histologically had Listeria in the blood. Bacteremia occurred in 2 (243, 263) shortly after inoculation and in 2 (861, 864) during the clinical stage of the disease.

The response of leukocytes in the blood was studied in 4 sheep (33, 243, 263, 864). Two of these had an early increase in neutrophils, which was associated with the recovery of Listeria from the blood. In 3 of the 4, the number of lymphocytes increased shortly after inoculation, but to a lesser degree than the neutrophils. The number of neutrophils increased or decreased sharply with the development of paralysis or paresis (fig. 1, 2).

Listeria agglutinins were present at dilutions of 1:4 to 1:64 in the serums of all sheep prior to inoculation. There was a subsequent rise in titers of two to eightfold in the majority of the test animals. There was no essential difference between preexposure titer or rise of titer that could be correlated with the subsequent outcome of the infection.

Listeria organisms were found primarily in the brain, spinal cord, and inoculated nerves in the 4 sheep which had listeriosis as defined by the criteria designated (fig. 3). The liver of 1 (243) was the only visceral organ in which Listeria were found. In 1 sheep (33), the bacteria were isolated from the root of the trigeminal nerve on the side opposite the superior buccal nerve that was inoculated.

Meningitis (pia-arachnoid) and encephalomyelitis were present in 6 sheep (33, 243, 677, 861, 864, 912). One (678) had encephalitis and 1 (865) had meningitis (pia-arachnoid) with myelitis. In those with meningo-encephalomyelitis, the necrotic foci, perivascular cuffing, and infiltration

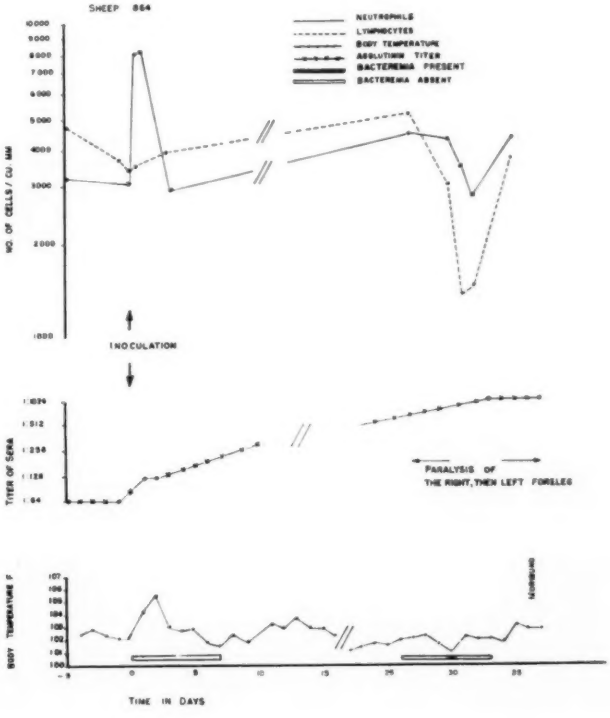

Fig. 1—Response of a sheep (864) to inoculation of 0.1 ml. of a 24-hour heart infusion broth culture of Listeria monocytogenes into the left infraorbital nerve.

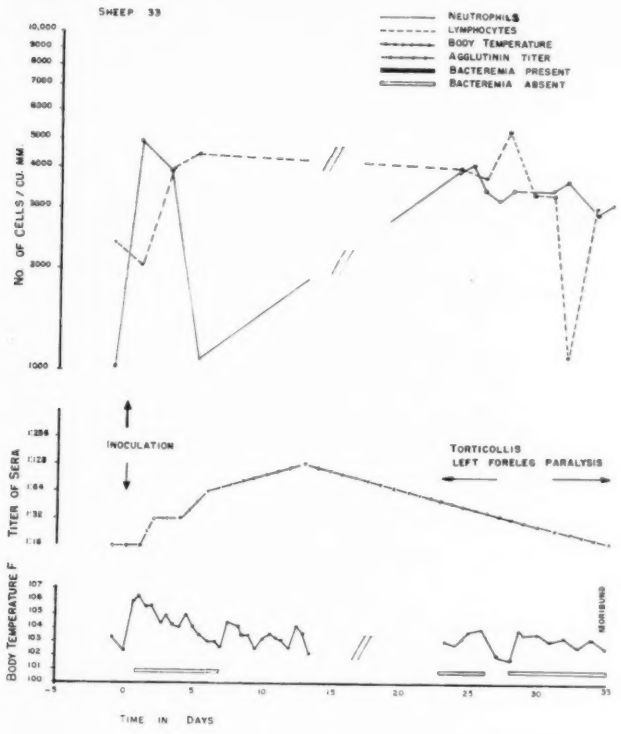

Fig. 2—Response of a sheep (33) inoculated in the left superior buccal nerve with approximately 0.1 ml of a 24-hour heart infusion broth culture of Listeria monocytogenes.

were confined mainly to the brain stem and spinal cord at the cervical and upper thoracic areas (fig. 4, 5, 6). The pons and medulla had marked cellular infiltration and necrotic foci in the reticular formation (fig. 4) and the adjacent ventral area in most cases.

Myelitis and radiculitis usually were restricted to the cervical and adjacent thoracic spinal cord. Infiltration of the dorsal root ganglia was found in 4 (33, 243, 861, 864) sheep. The perivascular reaction was most marked in the white matter of the cord (fig. 6). A frequent site of perivascular cuffing was in the area of the ventral motor nerve roots in the white matter of the cord. In general, the amount of cuffing and perivascular infiltration and the extent of necrosis increased from the midbrain through the brain stem to the spinal cord near the origin of the brachial plexus (fig. 6).

The subarachnoidal area of the leptomeninges of the brain was slightly infiltrated with histocytes, lymphocytes, and a few neutrophils. A larger number of these cells was found in the leptomeninges at the basal portion of the brain stem. There was, in some instances, continuity of the infiltrated area from the affected subarachnoid space to the perivascular areas of the spinal cord. Two sheep (243, 861) had a leptomeningitis which extended to the cauda equina.

The connective tissue sheaths of the inoculated nerves were infiltrated with variable numbers of lymphocytes, plasma cells, and neutrophils extending from the site of inoculation to the brain stems in 4 sheep (33, 243, 861, 912). In 2 sheep (678, 864), the reaction was localized at the site of inoculation. In two others (677, 865), no abnormal cellular infiltration was found in the facial or infraorbital nerves. The epineural and perineural sheaths of the left superior buccal, facial, infraorbital, and trigeminal nerves were infiltrated in sheep 33, which was inoculated in the left superior buccal nerve. Two sheep (243, 678) had cellular reactions in the epineural and perineural connective tissue in the left facial and superior buccal nerves, even though

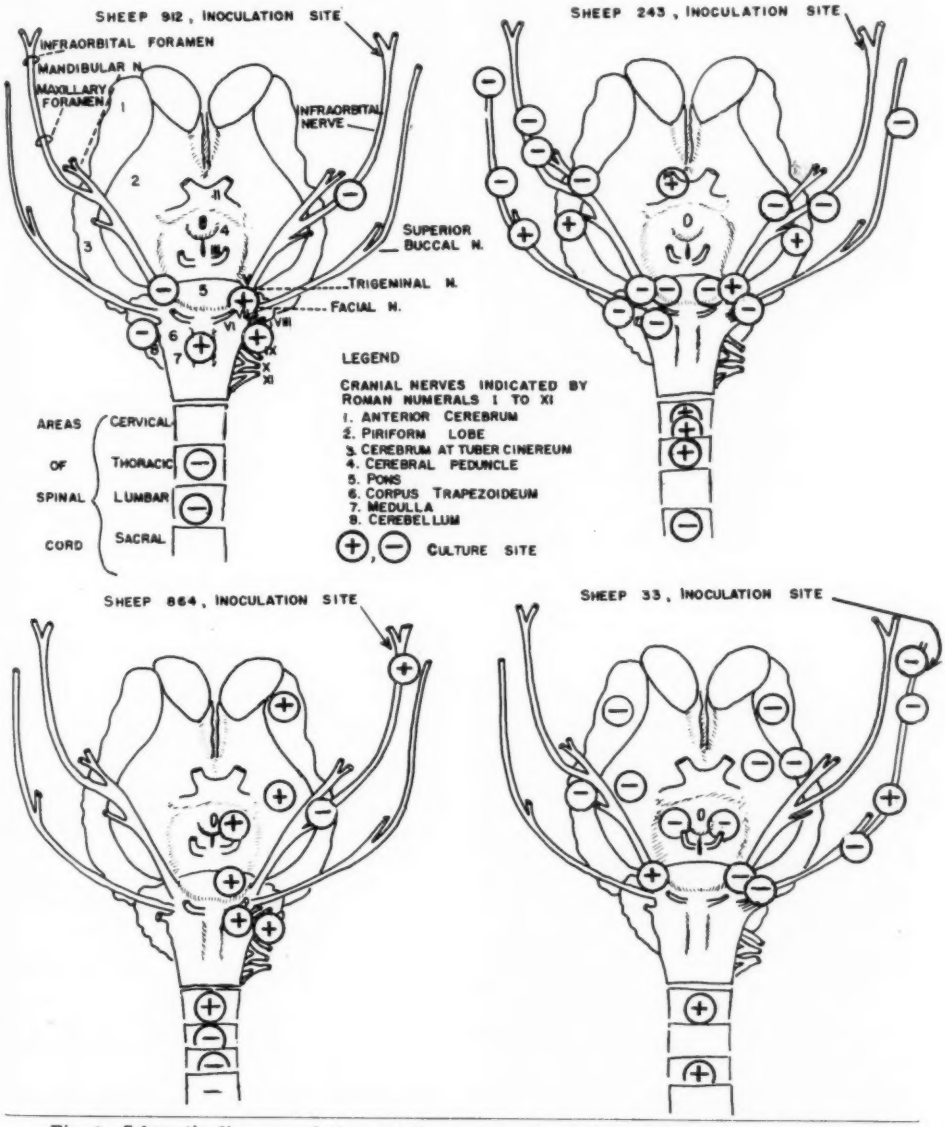

Fig. 3—Schematic diagrams of the central nervous system and cranial nerves of 4 sheep with listeriosis, indicating the place of inoculation and the sites where attempts were made to recover Listeria monocytogenes in culture.

they were inoculated in the left infraorbital nerves.

DISCUSSION

Of the 17 animals given direct inoculations in the branches of the trigeminal or facial nerves, 4 (24%) developed listeriosis with signs, lesions, and distribution of *L. monocytogenes* as seen in the naturally occurring disease. Asahi *et al.*[5] reported that 33 per cent (6 of 18) of the goats developed listeriosis after inoculation of Lis-

Fig. 4—Reticular area of pons of a sheep (33). Necrotic focus with infiltration of lymphocytes, plasma cells, a few neutrophils, and large numbers of pleomorphic histiocytic cells. Numerous cuffed vessels are seen in the adjacent area. H & E stain; orig. mag. x 27.

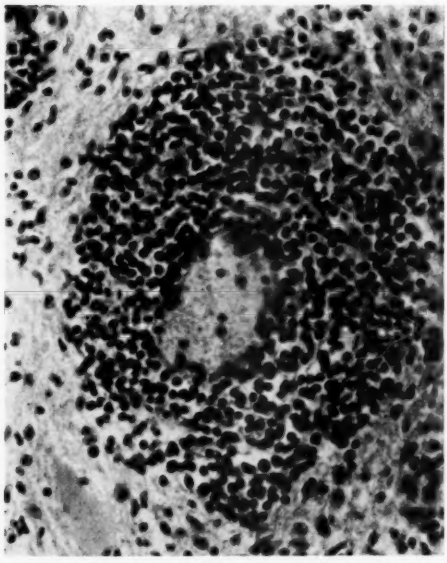

Fig. 5—Perivascular cuffing of a venule in the white matter of the spinal cord of sheep (33), at the level of the lower cervical vertebrae. There is hyperplasia of adventitial cells and cuffing with lymphocytes, plasma cells, and a few neutrophils. H & E stain; orig. mag. x 200.

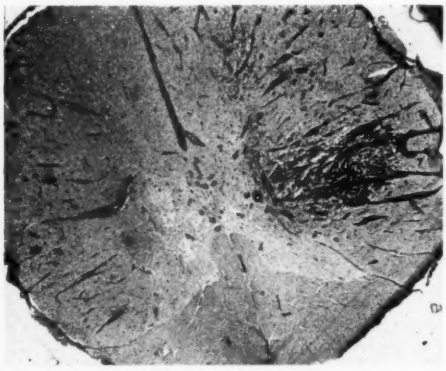

Fig. 6—Spinal cord at the level of the lower cervical vertebrae of a sheep (33). An unusually large focus of necrosis with infiltration of lymphocytes, plasma cells, and a few neutrophils is located in the lateral funiculus. Perivascular cuffing is extensive in the white matter and minimal in the grey matter and in the ventral fissure. H & E stain; orig. mag. x 13.

teria organisms into tissue areas innervated by these nerves. They stated that the microorganisms probably traveled along the trigeminal nerve to the brain stem.

In the present investigations and in those reported by Asahi *et al.*[3-5] the pathway of infection may have followed the connective tissue sheaths of the inoculated nerves to the pia-arachnoidal tissue or the subarachnoidal area of meninges of the brain and spinal cord. The organisms may then have entered the adjacent perivascular spaces and progressed into the deeper substance of the brain and spinal cord.

The histologic evidence for this hypothesis was supported by examination of tissue sections of the inoculated nerves and central nervous system. There was cellular reaction in the epineural, perineural, and endoneural connective tissue of these nerves. Necrotic foci and perivascular cuffing were prevalent in the basal portion of the pons and medulla. In the spinal cord, the perivascular reaction was most marked in the peripheral half; the most extensive necrotic foci were in the area of the spinal cord at the origin of the brachial plexus. The reaction to the infectious process occurred in those sites of the brain stem adjacent to the roots of the fifth and seventh cranial nerves. The subsequent progression of the infection may have been along the subarachnoidal area of the brain stem to the cervical and upper thoracic areas of the spinal cord. There is a limited amount of support for

this viewpoint. Asahi *et al.*[5] demonstrated a diffuse infiltration of the connective tissue sheaths of the trigeminal nerve bundles in mice and goats experimentally infected with Listeria. Histologic studies of the naturally infected sheep and goats also showed a "selective distribution of the lesions in the trigeminal nerve and its branches.[5]" Biester and Schwarte,[7] King,[18] and Cordy and Osebold [8] mentioned lesions of the trigeminal nerve in animals with naturally occurring cases. The number of sheep and goats examined was not stated in the first two reports. In the latter report, 4 of 15 sheep had nerve involvement. King [18] and Cordy and Osebold,[8] in their studies of naturally occurring cases of goats and sheep, respectively, did not report the extension of lesions to tracts of the trigeminal nerve, as reported by Asahi *et al.*[5]

An alternative pathway of infection to the central nervous system may be the hematogenous route. Cordy and Osebold [8] supported this hypothesis and, as evidence, suggested that the foci of necrosis arose at the termination of the cuffed arterioles, and that the earliest lesion in the brain was a local mobilization of microglial macrophages in the proximity of the capillaries. King [18] reported that, in goats, the cuffed vessels passed either directly through the necrotic foci or were in contact with the foci on one side. The latter detracts somewhat from this hypothesis. King [18] stated "sometimes, however, in spite of serial sections, a vascular relationship cannot be determined for every focus." Generally, in this study, the necrotic foci were adjacent to the area with the greatest concentration of heavily cuffed vessels. This fact indicated that the cuffing of the vessels followed as a reaction to the necrotic foci.

Cordy and Osebold [8] suggested that the necrotic foci preceded the vasomeningeal reaction by stating, "The vasomeningeal cellular infiltration with few or no bacteria suggests a mild injury by small numbers of bacteria, or more probably by irritant substances, draining centrifugally from deep foci into the cerebrospinal fluid." In their opinion, the vascular response was due to drainage of toxic substances from the necrotic foci. This idea would explain the absence of cuffing of vessels in areas of no or few necrotic foci. A further extension of this line of reasoning would lead to the assumption that the organism penetrated the brain substance prior to any cellular response around the blood vessels. Based on this explanation, Listeria may have reached the brain by way of the blood stream.

King [18] indicated that a vascular reaction had occurred by stating: "The coats of the blood vessels are profoundly disturbed. The endothelium may be swollen and proliferated. At other times it is degenerated and hyalinized. Small thrombi are not infrequent. The media may show marked disorganization and infiltration, with practically no structure remaining except infiltrating cells in an irregular hyaline matrix." It could be assumed that, if the route of infection is by way of the blood stream, the encephalitic form of listeriosis should be readily produced by intracarotid or intravenous inoculation. Listeria organisms, when given by these routes, are more likely to cause the generalized, acute form of the disease rather than the chronic encephalitic form.[11, 14, 19, 22]

Bacteremia occurred in 5 sheep, 4 of which developed the disease. There was an immediate increase in body temperature and numbers of circulating leukocytes following intraneural injection with Listeria. The agglutination titers rose during the course of the infection in most of the test sheep. The infiltration of the arachnoid villi in the superior sagittal sinus of 1 (243) suggested the possibility of the spread of the infection into the systemic circulation from the cerebrospinal fluid. These findings indicate a hematogenous involvement and systemic reaction at some time during the course of the infection.

The results of the present investigation are consistent both with the hematogenous theory advanced by King [18] and supported by Cordy and Osebold,[8] and with that supported by Asahi *et al.*, where the route of infection is thought to be along the pathways of cranial nerves. Perhaps the pathogenesis of encephalitic listeriosis may involve a combination of the nerve pathway and the hematogenous route.

SUMMARY

Four of 17 sheep given *Listeria monocytogenes* directly in the infraorbital or superior buccal nerves (branches of the trigeminal and facial nerves, respectively) developed listeriosis with localization of the infection in the central nervous system. Five other sheep had either lesions of the

central nervous system or clinical signs or both (2 sheep), suggesting infection.

These positive results may be interpreted as supporting the belief that infection occurs either by a hematogenous route or along the pathway of the trigeminal or facial nerves. The negative results (13 of 17 sheep) indicate that Listeria organisms do not always move from a peripheral branch of the trigeminal or facial nerves to the brain.

References

[1] Asahi, O., and Hosada, T.: Studies on Listeriosis in Domestic Animals. II. Studies on Experimental Listeriosis in Goats. Jap. J. Med. Sci. & Biol., 24, (1952): 38–41.

[2] Asahi, O., and Hosada, T.: Studies on Listeriosis in Domestic Animals. III. Studies on Experimental Listeriosis in Mice. Jap. J. Med. Sci. & Biol., 24, (1952): 100–103.

[3] Asahi, O., and Hosada, T.: Studies on Listeriosis in Domestic Animals. IV. Observation on the Route of Infection via Trigeminal Nerve Fiber of *Listeria monocytogenes* in Mice. Jap. J. Med. Sci. & Biol., 26, (1953): 72–75.

[4] Asahi, O., and Hosada, T.: Studies on Listeriosis in Domestic Animals. V. Inquiries Into the Mechanism of Spontaneous Infection of Listeriosis in Goats. Jap. J. Med. Sci. & Biol., 26, (1953): 196–199.

[5] Asahi, O., Hosada, T., and Akiyama, Y.: Studies on the Mechanism of Infection of the Brain with *Listeria monocytogenes*. Am. J. Vet. Res., 18, (Jan., 1957): 147–157.

[6] Asahi, O., Hosada, T., Akiyama, Y., and Ebi, Y.: Studies on Listeriosis in Domestic Animals. I. Listeriosis in Goats Found in Aomori Prefecture. Govt. Exper. Sta. for Anim. Hyg., Exptl. Rep., 27, (1945): 289–300.

[7] Biester, H. E., and Schwarte, L. H.: Studies of Listerella Infection in Sheep. J. Infect. Dis., 64, (1939): 135–144.

[8] Cordy, D. R., and Osebold, J. W.: The Neuropathogenesis of Listeria Encephalomyelitis in Sheep and Mice. J. Infect. Dis., 104, (1959): 164–173.

[9] Doyle, L. P.: Encephalitis in Sheep. J.A.V.M.A., 34, (July, 1932): 118–120.

[10] Gill, D. A.: "Circling Disease" of Sheep in New Zealand. Vet. J., 87, (1931): 60–74.

[11] Gill, D. A.: Circling Disease: A Meningo-Encephalitis of Sheep in New Zealand. Notes on a New Species of Pathogenic Organism. Vet. J., 89, (1933): 258–270.

[12] Graham, R.: Listerella Encephalitis or Encephalomyelitis in Domestic Animals. J.A.V.M.A., 95, (Sept., 1939): 289–292.

[13] Graham, R., Hester, H. R., and Levine, N. D.: Studies on Listerella. II. Field Outbreak of Listerellosis in Sheep and Cattle. Cornell Vet., 30, (1940B): 97–111.

[14] Graham, R., Levine, N. D., Morrell, C. D.: Listerellosis in Domestic Animals. University of Illinois Agric. Exper. Sta. Bull. 499, 1943.

[15] Gray, M. L.: A Rapid Method for the Detection of Colonies of *Listeria monocytogenes*. Zentralbl. Bakt., 169, (1957): 373–377.

[16] Gray, M. L., Stafseth, H. J., Thorp, F., Jr., Holland, L. B., and Riley, W. F., Jr.: A New Technique for Isolating Listerella from the Bovine Brain. J. Bact., 55, (1948): 471–476.

[17] Jungherr, E.: Ovine Encephalomyelitis Associated with Listerella Infection. J.A.V.M.A., 91, (July, 1937): 73–87.

[18] King, L. S.: Primary Encephalomyelitis in Goats Associated with Listerella Infection. Am. J. Path., 16, (1940): 467–478.

[19] Olafson, P.: Listerella Encephalitis (Circling Disease) of Sheep, Cattle, and Goats. Cornell Vet., 30, (1940): 141–150.

[20] Olson, C., Dunn, L. S., and Rollins, C. L.: Methods for Isolation of *Listeria monocytogenes* from Sheep. Am. J. Vet. Res., 15, (Jan., 1953): 82–85.

[21] Olson, C., Grace, O. D., Segre, D., and Blore, I. C.: Enhancement of Listeriosis in Sheep with Material from Bovine Mucosal Disease. Am. J. Vet. Res., 18, (April, 1957): 303–309.

[22] Olson, C., Rollins, C. L., Bagdonas, V., Blore, I. C., and Segre, D.: The Distribution of *Listeria monocytogenes* in Listeriosis of Sheep. J. Infect. Dis., 93, (1953): 247–256.

[23] Pallaske, G.: Weitere Untersuchungen über die Listerella besonders beim Tier. Zentralbl. Vet.-med., Suppl. No. 1, (1958): 49–58.

[24] Paterson, J. S.: Studies on Organisms of the Genus Listerella. IV. An Outbreak of Abortion Associated with the Recovery of Listerella from the Aborted Fetuses. Vet. J., 96, (1940): 327–332.

SUMMARIO IN INTERLINGUA

Le Nervos Trigeminal e Facial Como Vias de Infection per *Listeria monocytogenes* in Oves

Quatro de 17 oves recipiente *Listeria monocytogenes* directemente in le nervos infraorbital o supero-buccal (que es brancas del nervos trigeminal e facial, respectivemente) disveloppava listeriosis con localisation del infection in le systema nervose central. Cinque altere oves habeva lesiones del systema nervose central e/o signos clinic que suggereva le presentia de un infection. In duo del cinque, lesiones e signos esseva constatate.

Iste positive resultatos pote esser interpretate como corroboration del notion que le infection occurre via le sanguine o via le nervos trigeminal o facial. Le negative casos (13 ex 17) indica que le organismo in question non migra invariabilemente ab un branca peripheric del nervos trigeminal o facial verso le cerebro.

Electrocardiographic and Phonocardiographic Patterns of Normal Lambs

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COMPARED to the electrocardiographic information available on adult sheep,[8-11,13] only scanty data exist on electrocardiography in lambs.[1,7] In both adult and young sheep, all of the electrocardiographic values have been limited to the standard or bipolar limb leads; unipolar and thoracic leads have not been reported. The literature contains reports of phonocardiography in only 2 adult sheep.[9] As the result of an almost complete absence of information on electrocardiography and phonocardiography in the young lamb, this study was conducted to determine the normal patterns in animals 1 to 4 weeks of age.

MATERIALS AND METHODS

Electrocardiographic and phonocardiographic tracings were taken on 92 lambs (both sexes) without the use of sedatives or tranquilizers. All animals (white-faced native-Hampshire crossbred) were raised at an elevation of 5,000 ft. above sea level on the University Experimental Farm. The number of lambs available for the study at 1, 2, 3, and 4 weeks of age were 13, 27, 20, and 32, respectively. Prior to each recording, the animals were restrained comfortably in a canvas sling with their feet hanging free through properly placed openings in the canvas support. Hypodermic needles (20-gauge, 1 inch) inserted subcutaneously into the limbs were used for electrodes. The needles were placed vertically and laterally in the olecranon region and opposite the patella paralleling the straight patellar ligament. Thoracic lead needles were positioned subcutaneously over the midcardiac region of the right (V_1) and left (V_3) fourth intercostal spaces. The midline thoracic lead (V_2) was inserted horizontally in the transverse plane under the sternum, just anterior to the xiphoid region.

Phonocardiographic tracings (set at the stetho position) were taken simultaneously with the bipolar limb lead recordings. The microphone was held in place at the fourth intercostal space over the midcardiac region on the left side.

All tracings, including those of the unipolar and thoracic leads, were recorded by a Model 62 Twin Beam Sanborn electrocardiograph and phonocardiograph.* The tracings were obtained on photographic film traveling at a speed of 75 mm. per second. Each recording was analyzed with calipers to determine the intervals (in milliseconds) of the various tracing components by obtaining the average of five readings from each lead. Calibration (1 mv.) of the electrocardiograph was carried out for each lead recorded. The nomenclature for analyzing the tracings was in accordance to that described by the American Heart Association.[3]

RESULTS AND DISCUSSION

The values obtained in this study (tables 1–4) and representative electrocardiograms and phonocardiograms (fig. 1–4) for lambs 1 to 4 weeks of age are given.

In this study, tracings were made from the standard limb or bipolar leads, unipolar (aVr, aVl, and aVf) leads, and thoracic (V_1, V_2, and V_3) leads. Only three thoracic leads were feasible, due to the relatively small size of the thorax. The unipolar and thoracic leads are valuable in detecting the electrocardiographic cardiac position and in understanding changes resulting from myocardial infarction, hypertrophy, bundle branch block, premature contractions, and other cardiac abnormalities.[2]

The electrocardiographic data reported by Kelso[7] for normal Merino lambs (1 to 12 weeks of age) differed in many respects from those obtained in this study. For example, the amplitude of some of the deflection waves varied markedly. Some of these differences probably can be attributed to factors such as the breed and sex of the animal, plane of nutrition, metabolic activity,[12] and the environment to which the animal is subjected. Moreover, the type of instrument used in recording elec-

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The data herein were taken from the thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. degree.

* Manufactured by the Sanborn Co., Cambridge, Mass.

TABLE 1—Heart Rate and Heart Sounds Recorded from Lead II on Lambs 1 to 4 Weeks of Age

Age (weeks)	Heart rate per minute		Duration of heart sounds (millisec.)				No. of vibrations of each heart sound			
			First sound		Second sound		First sound		Second sound	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1	206.5	135–270	49.0	40–60	24.0	15–30	4.5	3–6	2.3	2–3
2	190.3	150–270	55.0	30–70	25.0	15–40	5.4	3–7	2.5	2–4
3	164.8	100–260	56.0	40–80	32.0	20–40	5.8	4–7	2.9	2–4
4	141.1	105–200	68.0	50–90	36.0	20–50	6.1	4–8	3.3	2–5

trocardiographic data is of importance. The tracings obtained from a direct-writing electrocardiograph as used by Kelso would be somewhat less accurate than those obtained by a photographic electrocardiograph. The photographic instrument used in this study is capable of following the electrical outflow from the heart more readily, since it possesses a higher natural frequency than a direct-writing instrument.

Upon comparing the results from the standard limb leads of this study with those of Kelso,[7] it was observed that the P-R intervals of the two studies differed. In Kelso's work, the P-R intervals varied considerably for lambs 1 to 4 weeks of age; whereas, in this study, the P-R intervals increased progressively with the age of the animal. In adult animals, the presence of a longer P-R interval bears direct evi-

TABLE 2—The P-R Intervals (in milliseconds) of Lambs 1 to 4 Weeks of Age

Lead	1 week old		2 weeks old		3 weeks old		4 weeks old	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
I	70	55– 80	72	50– 90	76	55– 95	80	....–110*
II	71	40– 90	75	65–100	77	60–100	91	70–120
III	60	40– 90	67	....– 80	80	55– 90	87	70–120
aVr	75	60–120	75	60–100	83	60– 95	90	55–130
aVl	68	50– 80	72	40– 95	70	60– 90	88	70–105
aVf	73	55– 90	77	55–110	80	65–100	89	60–120
V_1	70	55–100	68	60– 80	77	....– 90	83	....–120
V_2	76	60–120	76	....–100	82	70– 95	89	70–120
V_3	71	60–100	75	50–100	81	65– 95	88	70–120

* .... = absence of interval; *i.e.*, presence of flat P wave.

It is interesting that the deflection waves of adult sheep, recorded by Souza [13] from the standard limb leads, were less than those recorded for lambs. This was obviously true of the deflection waves recorded in lead II tracings from the lambs of this study.[6] According to Luisada,[8] tracings obtained on aged human subjects may reveal lower voltage waves than those on younger people.

dence of this observation.[13] According to Kelso, the P-R interval was greatest in lead III and nearly equal in the remaining bipolar leads. However, in this study, the average P-R interval for 1-, 2-, and 4-week old lambs was greater in lead II than in leads I and III.

The Q-T intervals in this study and in the study of Kelso [7] increased progressively with the age of the animal. This trend was

TABLE 3—The QRS Intervals (in milliseconds) of Lambs 1 to 4 Weeks of Age

Lead	1 week old		2 weeks old		3 weeks old		4 weeks old	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
I	30	20–40	32	15–40	34	25–40	32	20–40
II	25	20–40	28	15–40	32	20–45	33	20–50
III	27	20–40	28	15–40	35	25–55	35	25–40
aVr	27	15–40	30	20–50	30	15–45	33	25–50
aVl	26	20–40	26	20–40	30	20–45	36	30–50
aVf	26	20–40	28	10–50	33	20–55	35	25–45
V_1	25	20–40	33	20–50	35	25–45	39	25–55
V_2	28	20–40	37	25–55	35	25–45	41	30–60
V_3	25	10–40	28	20–40	33	20–40	35	25–55

TABLE 4—The Q-T Intervals (in milliseconds) of Lambs 1 to 4 Weeks of Age

Lead	1 week old		2 weeks old		3 weeks old		4 weeks old	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
I	121	.....–200*	173	.....–200	114	.....–225	125	.....–240
II	171	140–200	163	.....–210	197	145–230	218	190–240
III	170	140–220	198	120–210	200	150–230	214	180–250
aVr	168	100–220	176	130–210	203	150–230	219	170–240
aVl	157	120–220	157	.....–210	169	.....–230	209	.....–240
aVf	171	130–230	176	135–210	190	170–240	223	190–245
V_1	162	120–220	164	.....–200	176	.....–240	205	.....–240
V_2	169	130–210	182	140–230	203	170–240	223	170–250
V_3	173	150–220	171	.....–210	189	.....–235	212	.....–255

* ..... = absence of the interval; *i.e.*, presence of flat T wave.

seen in Souza's studies [13] on adult sheep, where the interval was nearly three times that of the lamb. In Kelso's study, the QRS intervals appeared to be unrelated, regardless of the age of the lambs. In this study, the interval gradually increased with age. No comparison could be made between this study and that of Baeigalupo *et al.*,[1] because their data were averaged for all animals over 12 weeks of age. Except in leads V_1 and V_2, QS waves were occasionally present. Some R prime waves were erratically seen in all leads and S prime waves were seen on various occasions in all leads except II, III, and aVf.

The heart rates in this study and in that of Kelso [7] decreased with age. These observations are in agreement with those of Dukes.[4] The more rapid heart rate in young animals is explained in part by their smaller size plus the fact that tonic vagal inhibition is less well developed in young animals.

As far as could be determined from a survey of the literature, this study is the first one to obtain phonocardiographic tracings on lambs. The only phonocardiographic information available on sheep has been that obtained by Luisada *et al.* on 2 adult animals. As was expected, the phonocardiographic tracings recorded on lambs were distinctly different from those recorded by Luisada *et al.* on mature sheep.

It is known that the vibrations and durations of the heart sounds are not only variable with age but may be variable within the same subject.[5] In lambs, the first sound consisted of three to seven large vibrations followed by one to two small vibrations. The second heart sound consisted of two to three quick vibrations which were sometimes followed by one slow wave. For the first heart sound in adult sheep, Luisada *et al.* recorded one large vibration preceded and followed by two to four smaller vibrations and one to two quick vibrations for

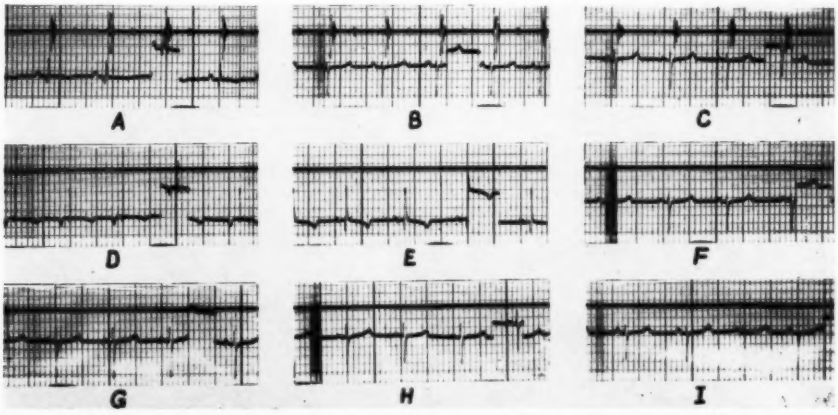

Fig. 1—Representative electrocardiogram and phonocardiogram of a 1-week-old lamb. (A) lead I; (B) lead II; (C) lead III; (D) aVr lead; (E) aVl lead; (F) a Vf lead; (G) V_1 lead; (H) V_2 lead; (I) V_3 lead. The standardization wave is 1 mv. and the unit of time is 40 milliseconds.

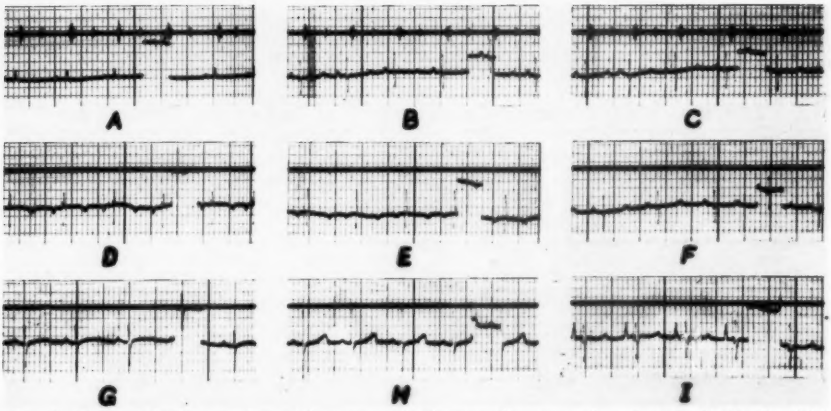

Fig. 2—Representative electrocardiogram and phonocardiogram of a 2-week-old lamb. (A) lead I; (B) lead II; (C) lead III; (D) a Vr lead; (E) aVl lead; (F) a Vf lead; (G) V_1 lead; (H) V_2 lead; (I) V_3 lead. The standardization wave is 1 mv. and the unit of time is 40 milliseconds.

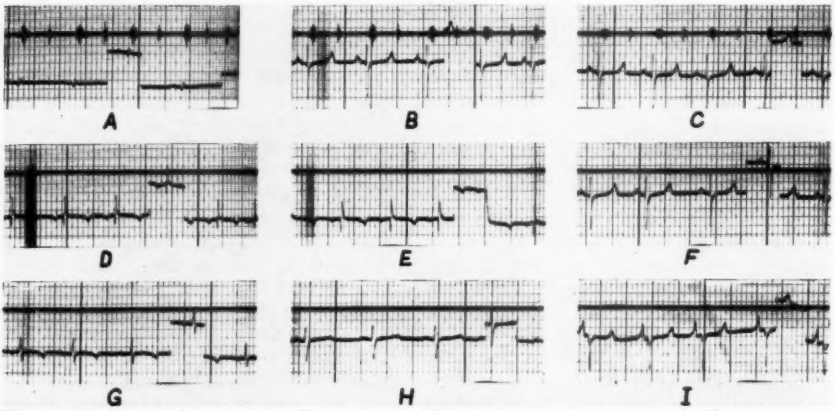

Fig. 3—Representative electrocardiogram and phonocardiogram of a 3-week-old lamb. (A) lead I; (B) lead II; (C) lead III; (D) aVr lead; (E) a Vl lead; (F) a Vf lead; (G) V_1 lead; (H) V_2 lead; (I) V_3 lead. The standardization wave is 1 mv. and the unit of time is 40 milliseconds.

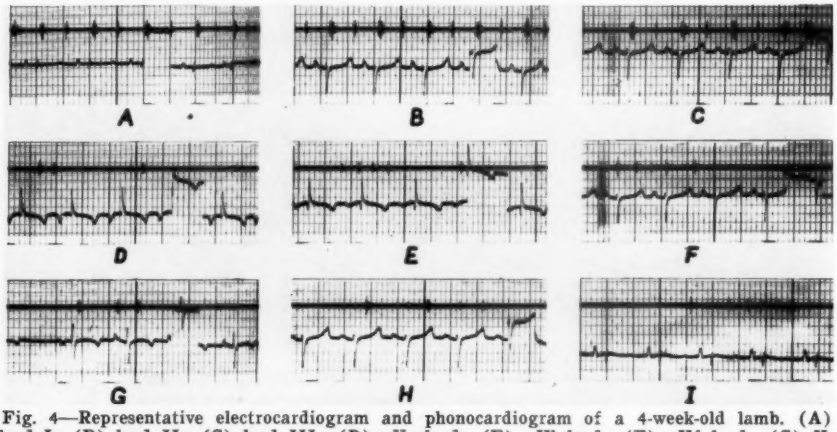

Fig. 4—Representative electrocardiogram and phonocardiogram of a 4-week-old lamb. (A) lead I; (B) lead II; (C) lead III; (D) aVr lead; (E) aVl lead; (F) aVf lead; (G) V_1 lead; (H) V_2 lead; (I) V_3 lead. The standardization wave is 1 mv. and the unit of time is 40 milliseconds. The phonocardiograph microphone sensitivity control was inadvertently left on during the recording of tracings D, E, F, G, H, and I. The deflections noticed in each tracing are artifacts produced by extraneous noises.

the second sound. The duration of time recorded for both first and second sounds in adult sheep was longer than that recorded for lambs. This would be expected, since the duration of the heart sounds for lambs in this study increased progressively with age.

SUMMARY

Electrocardiographic and phonocardiographic patterns and ranges of values were determined in 92 lambs from 1 to 4 weeks of age. The standard (or bipolar), unipolar, and thoracic leads were recorded in all of the animals without use of sedatives or tranquilizers. It was found that the P-R, QRS, and Q-T intervals increased progressively with age, and that heart rates decreased. Insofar as is known, this is the first study reporting the electrocardiographic recording of unipolar and thoracic leads and also represents the first time that phonocardiograms have been recorded in the young lamb.

References

[1] Bacigalupo, F. A., Alfredson, B. V., Luecke, R. W., and Thorp, F.: Electrocardiographic Changes in Vitamin E Deficient Lambs. Am. J. Vet. Res., 14, (April, 1953): 214–218.

[2] Burch, G. E., and Winsor, T.: A Primer of Electrocardiography. 3rd ed. Lea & Febiger, Philadelphia, (1945): 109–133.

[3] Barnes, A. R., Katz, L. N., Levine, S. A., Pardee, H. E. B., White, P. D., and Wilson, F. N.: Report of the Committee of the American Heart Association on the Standardization of Electrocardiographic Nomenclature. Am. Heart J., 25, (1943): 528–534.

[4] Dukes, H. H.: The Physiology of Domestic Animals. 7th ed. Comstock Publishing Assoc., Ithaca, N.Y., (1955): 142–143.

[5] Fahr, G.: On Simultaneous Records of the Heart Sounds and the Electrocardiogram. Heart, 4, (1912): 147–170.

[6] Hilmy, M. I.: Electrocardiographic and Phonocardiographic Patterns of Lambs. M.S. thesis, Colorado State University, Fort Collins, 1957.

[7] Kelso, W. T.: Electrocardiograms of Young Lambs. Queensland Agric. Sci., 4, (1947): 60–77.

[8] Luisada, A. A.: The Heart Beat. Paul B. Hoeber, Inc., New York, N.Y., (1953): 292.

[9] Luisada, A. A., Weisz, L., and Hantman, H. W.: A Comparative Study of Electrocardiogram and Heart Sounds in Common and Domestic Mammals. Cardiologia, 8, (1944): 60–84.

[10] Mullick, D. N., Alfredson, B. V., and Reineke, E. P.: Influence of Thyroid Status on the Electrocardiogram and Certain Blood Constituents of the Sheep. Am. J. Physiol., 152, (1948): 100–105.

[11] Platner, W. S., Kibler, H. H., and Brody, S.: Growth and Development. LXIII. Electrocardiograms of Mules, Horses, Cattle, Sheep, Swine, and Goats. Missouri Agric. Exper. Sta. Res. Bull. 419, (1948): 1–12.

[12] Robb, J. S.: A Study of Q-T Interval in Various Species. Fed. Proc., 5, (1946): 87.

[13] Souza, R. De.: The Action of Anesthetics and Ions on the Heart of the Sheep as Revealed by the Electrocardiogram. M.S. thesis, Cornell University, Ithaca, N.Y., 1952.

SUMMARIO IN INTERLINGUA

Configurationes Electro- e Phonocardiographic del Agno Normal

Le configurationes electro- e phonocardiographic e le variabilitate del valores esseva determinate pro 92 agnos de etates de inter 1 e 4 septimanas. Le derivationes standard (o bipolar), unipolar, e thoracic esseva registrate in omne le animales sin le uso de sedativos o tranquillisantes. Esseva constatate que le intervallos P-R, QRS, e Q-T cresceva progressivemente con le avanciamento del etate e que le frequentia cardiac decresceva. In tanto que nos lo sape, isto es le prime studio reportante le registration electrocardiographic de derivationes unipolar e thoracic in agnos. Isto es etiam le prime vice que phonocardiogrammas es obtenite in agnos de basse etate.

XUM

Staphylococcic Antitoxins in Dairy Cattle. I. A Review of the Literature

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INTEREST IN bovine staphylococcic mastitis has increased in recent years, and it is anticipated that much more research will be done in this field. The data are summarized in this review article on staphylococcic antitoxins in the blood, milk, and colostrum of dairy cattle.

METHOD OF TABULATION

The principal observations presented in this review are summarized in tables. Three levels of antitoxin titers were chosen for grouping the cattle, since the published data could be used with the following minor changes:

1) All cattle listed by Minett[11] as having titers less than 1.1, 1.2, or 1.5 international units (I.U.) per milliliter of serum were recorded as less than 1.0 I.U. in tables 1 and 3. In addition, those animals that were listed as having titers of 2.3 to 4.6 I.U. were placed in the 1.0 to 4.0 I.U. group.

2) In tables 1 and 2, all cattle listed by Ramon *et al.*[15-20] and Richou *et al.*[21, 22, 24, 26] as having titers of 3.0 to 5.0 I.U. were placed in the 1.0 to 4.0 I.U. group. Only the antitoxin titers of serum samples tested by these workers before injection of toxoid were used in these tables.

3) Only 6 of the 18 animals studied by Richou *et al.*[22] are listed in table 1. The other 12 animals are listed in table 1 under Richou and Holstein,[21] who had used the same animals in their table 3.

4) The animals listed as heifers and young steers by Gwatkin[5] and those listed as heifers by Thörne[31] are listed in table 2. It was assumed that these animals were less than 2 years old.

5) The data in figure 1 were obtained from table 4 by Minett.[11] All animals listed by Minett as having less than 1.2 I.U. per milliliter of serum were considered as having 0.0 I.U., for purposes of calculating the average titers. In addition, those animals that had 2.3 to 4.6 I.U. were considered as having 3.4 I.U., and those that had 15.0 I.U. or more as having 15.0 I.U.

REVIEW

Alpha Antitoxin in Blood Serum.—Staphylococcic *alpha* antitoxin has been found in the blood of cattle by Ramon *et al.*,[15-20] Minett,[9, 11] Gwatkin,[5] Richou *et al.*,[21, 22, 24-26] Miller and Heishman,[8] Lasmanis and Spencer,[7] Thörne, [31] Slanetz *et al.*, [27] and Edwards and Smith.[3]

The results reported by these workers indicate that staphylococcic *alpha* antitoxin is present in a large percentage of cattle and that the serum titers may show wide variations. Workers have shown that the incidence and the amount of *alpha* antitoxin in blood serums are higher in older cattle, although the relationship is not exact because of individual differences.[3, 11, 16, 24] A comparison of data in tables 1 and 2 shows the effect of age on the level of antitoxin titer. In cattle 2 years of age or older (table 1), about 63 per cent had serum titers of at least 1 I.U., as compared to 20 per cent of the cattle less than 2 years of age (table 2). Furthermore, 27 per cent of the older cattle had titers of more than 4 I.U. as compared to only 2 per cent of the younger group. Ramon *et al.*[16] reported that the average amount of antitoxin in the serums of female cattle was about 1 I.U. for the animals 5 to 8 months of age, more than 1 I.U. for animals 1 to 2 years of age, and more than 2 I.U. for those older than 2 years. Ramon *et al.*[16] also demonstrated *alpha* antitoxin in the blood of calves at birth, although the titers were less than the titers of the dams. It was not stated if the blood samples were taken before or after the calves nursed. Apparently no consideration was given to the calf obtaining antibodies by ingestion of colostrum; consequently, we feel they wrongly explained the presence of the antitoxin as a passive immunity transmitted from the mother to the fetus through the placenta. Edwards and Smith[3] found no antitoxin at birth before nursing but, at 2 days of age, all of the 26 calves tested had serum titers, generally at a low level. The persistence of titer was directly related to the titer of the dam's blood and colostrum.

One reason for the higher level of serum *alpha* antitoxin in the older animals is the

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influence of udder infection with toxigenic staphylococci.[3, 5, 7, 8, 11, 31] The relationship between higher antitoxin titers and udder infection can be seen (table 3). In the cows with infected udders, 90 per cent had serum titers of at least 1 I.U. as compared to 57 per cent of the cows with noninfected udders. According to Miller and Heishman,[8] the relationship between higher titers and infection was not complete in the animals they studied until the titer rose above 16 I.U. Minett[11] found, however, that the increase in titer with age occurred in bulls and in cows regardless of the presence or absence of staphylococcic udder infections. Thus, infections with toxigenic staphylococci in areas other than the udder may also be responsible for increased titers in older cattle.[3, 8, 11, 31] To better illustrate the combined effect of age and staphylococcic udder infection on the level of serum *alpha* antitoxin, data presented by Minett[11] have been reworked and presented graphically (fig. 1). Minett[11] also found that in cows with staphylococci-infected udders the antitoxin titers remained rather constant, except after calving when an increase occurred in some cows. He attributed this to renewed growth of staphylococci in the refunctioning udder. Edwards and Smith[3] also found after calving that an increase in titer developed primarily as a result of clinical mastitis caused by staphylococci. Only 8 of 77 cows showed an increased titer and, of these, 5 developed clinical mastitis. It was not stated, however, how many of the 77 cows had staphylococcic udder infections.

Another factor which may influence the blood antitoxin titer is the number of quarters infected. As seen in data on 34 cows with staphylococci-infected udders, the blood *alpha* and *beta* antitoxin and agglutinin titers tended to increase with the number of infected quarters.[1] Slanetz[28] and Edwards[4] have kindly made available to us unpublished data on 149 cows and 38 cows, respectively, with staphylo-

TABLE 1—Blood Serum Alpha Antitoxin Titers in Cattle Two Years of Age and Older

Author	Year	No. of cattle grouped according to titers in I.U.*			Total No. of cattle
		<1	1 to 4	>4	
Ramon *et al.*[15]	1935	7	4	0	11
Gwatkin[5]	1937	0	6	14	20
Minett[11]	1937	229	226	112	567
Richou and Holstein[21]	1941	4	12	0	16
Richou *et al.*[22]	1941	2	3	1	6
Miller and Heishman[8]	1943	69	98	53	220
Ramon *et al.*[16]	1951	31	30	5	66
Ramon *et al.*[17]	1952	7	2	0	9
Ramon *et al.*[18]	1952	10	4	0	14
Ramon *et al.*[19]	1952	88	77	5	170
Richou *et al.*[24]	1952	37	35	2	74
Richou *et al.*[26]	1952	1	7	2	10
Ramon *et al.*[20]	1953	9	43	8	60
Lasmanis and Spencer[7]	1954	36	13	15	64
Thörne[31]	1958	122	50	19	191
Slanetz[28]	1959	80	68	282	430
Edwards[4]	1959	47	76	57	180
Total		779	754	575	2,108
Percentage of total		36.9	35.8	27.3	.....

* Titers are given in international units per milliliter of serum.

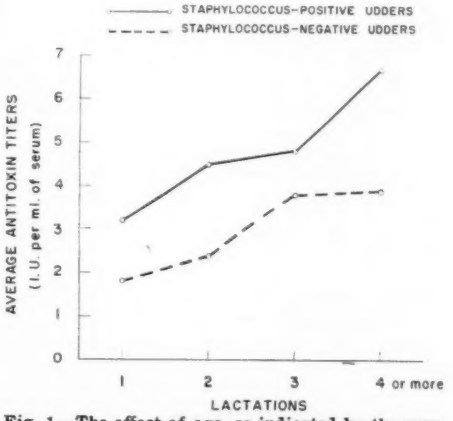

Fig. 1—The effect of age, as indicated by the number of lactations, and staphylococcic udder infection on the blood serum alpha antitoxin in dairy cows (plotted from data in table 4 by Minett[11]).

TABLE 2—Blood Serum Alpha Antitoxin Titers in Cattle Less Than Two Years Old

Author	Year	No. of cattle grouped according to titers in I.U.*			Total No. of cattle
		< 1	1 to 4	> 4	
Gwatkin [5]	1937	21	17	4	42
Ramon *et al.*[16]	1951	39	19	0	58
Ramon *et al.*[18]	1952	13	2	0	15
Ramon *et al.*[19]	1952	19	0	0	19
Richou *et al.*[24]	1952	19	2	0	21
Ramon *et al.*[20]	1953	4	1	0	5
Thörne [31]	1958	48	2	0	50
Edwards [4]	1959	22	0	0	22
Total		185	43	4	232
Percentage of total		79.8	18.5	1.7	.....

* Titers are given in international units per milliliter of serum.

cocci-infected udders. The combined data from these three sources (table 4) indicate the relationship between the level of blood *alpha* antitoxin and the number of infected quarters. The relationship is not exact when individual animals are compared. The average antitoxin titers for cows of each group also varied in the three investigations. The cows studied by Brown [1] had average antitoxin titers which ranged from 10.6 I.U. for those infected in one quarter to 25.8 I.U. for those infected in four quarters. For the cows studied by Slanetz, the average antitoxin titers ranged from 8.3 I.U. for those infected in one quarter to 28.5 I.U. for those infected in four quarters; whereas, those studied by Edwards ranged from 3.8 I.U. to 10.5 I.U.

TABLE 3—The Relationship of Udder Infection to the Presence of Alpha Antitoxin in the Blood

Author	Year	Staphylococcus-positive (No. of cows grouped according to titers in I.U.*)			Staphylococcus-negative (No. of cows grouped according to titers in I.U.*)		
		< 1	1 to 4	> 4	< 1	1 to 4	> 4
Gwatkin [5]	1937	0	3	12	0	3	2
Minett [11]	1937	28	103	55	74	81	36
Miller and Heishman [8]	1943	8	47	37	61	51	16
Lasmanis and Spencer [7]	1954	2	7	11	34	6	4
Thörne [31]	1958	5	16	19	83	22	0
Slanetz [28]	1959	9	16	124	71	52	158
Edwards [4]	1959	7	40	28	15	11	4
Total		59	232	286	338	226	220
Percentage of total		10.2	40.2	49.6	43.1	28.8	28.1
		Total = 577			Total = 784		

* Titers are given in international units per milliliter of serum.

Alpha Antitoxin in Milk and Colostrum.—Alpha antitoxin has been found in milk whey under natural conditions [3, 5, 7, 8, 11, 31] and after the cow had been given a parenteral injection of toxoid.[11, 21, 29] The findings indicate that the antitoxin titer for milk is generally much less than the antitoxin titer for blood.[3, 5, 7, 8, 11, 31] Minett found that, in two thirds of 331 whey samples from normal quarters, the *alpha* antitoxin content was from 1/80 to 1/40 of that in the blood serum.[11] Although the whey/serum antitoxin ratio generally increased as the blood titers increased, a conversion of the ratios to units of antitoxin in whey showed that the whey titers tended to be higher as the blood titers increased. Edwards and Smith [3] also found a definite relationship between the blood and milk antitoxin titers. In 46 cows with blood titers of 1.0 to 4.0 I.U. of antitoxin, only 3.2 per cent of the quarters had milk titers of 0.2 I.U. or more; whereas, in 14 cows with blood titers of 8.0 to 32.0 I.U., 64 per cent of the quarters had milk titers of 0.2 I.U. of antitoxin or more. The concentration of antitoxin also appears to be correlated with the pH of the milk.[7] Milk with increased alkalinity generally contains larger amounts of antitoxin.[3, 7, 11] Thus, staphylococci-infected quarters tend to have higher whey titers than noninfected quarters.[3, 5, 7, 8, 11, 31] Milk from streptococci-

infected quarters has also been found to have elevated antitoxin titers.[3,11] According to Lasmanis and Spencer,[7] the whey titers probably depend upon the serum titer and the permeability of the mammary tissues that are interposed between the blood and the milk. Consequently, any disturbance capable of causing inflammation in the quarter could increase the whey titer. The source of antibodies in milk is discussed by Minett[11] and Kerr *et al.*[6]

Minett,[11] Miller and Heishman,[8] and Edwards and Smith[3] found that the concentration of antitoxin in colostrum may equal or exceed, by four to six times, that found in the blood of the same animal. The titer drops rapidly after parturition, however, and may be absent in the milk several days to several weeks later. The titers persist longer and at a higher level in the milk of cows that have developed clinical mastitis.[3]

Kerr *et al.*[6] reported that the infusion of toxoid into the lactating udder resulted in severe reactions, but no antitoxin was detected in the milk. When the cows entered the dry phase, antitoxins were found in the blood serum and in the udder secretion. The workers considered these to be serum-globulin antitoxins, since they appeared equally in all quarters and disappeared after the colostral phase. This was not the case when the antibodies were produced locally against other antigens. These workers believed that detectable amounts of antitoxin were not produced in the lactating udder against staphylococcic toxoid. Taylor[30] also found that infusion of the udder with Staphylococcus toxin or toxoid produced a local inflammatory reaction. Contrary to the results of Kerr *et al.*,[6] however, Taylor found that antitoxin was produced in the udder. After infusion of a quarter, antitoxin was found in the milk of the treated quarter first and later in the blood and milk of the uninoculated quarters. The level of antitoxin in the blood, however, became higher than in the milk of any of the quarters.

Beta Antitoxin in Blood Serum.—*Beta* antitoxin has been demonstrated in the blood serum of cattle, but at a lower incidence than *alpha* antitoxin.[3,9,11,17,20,24-26] In 95 cattle tested by Richou *et al.*,[24] the incidence of the two antitoxins in the blood was 84 per cent for *alpha* and 74 per cent for *beta*. Likewise, in 202 cattle tested by Edwards and Smith,[3] the incidence of antitoxins was 79 per cent for *alpha* and 59 per cent for *beta*. Workers of both groups also found that the incidence and amount of *beta* antitoxin generally increased with age. The *beta* antitoxin titers are also usually higher in cows with staphylococci-infected udders and have a relationship analogous to that of the *alpha* antitoxin listed in table 4.[1]

Beta Antitoxin in Milk and Colostrum.—*Beta* antitoxin has been found in milk and colostrum by Edwards and Smith.[3] The titer is elevated in colostrum and declines after calving in a manner similar to that found for *alpha* antitoxin. The data as presented in their paper did not make it clear if there was a relationship between the milk titer and udder infection, alkalinity of the milk, or blood titer, as was the case for *alpha* antitoxin.

Toxoid Injection.—The intramammary injection of a staphylococcic culture,[8,11] as well as the subcutaneous or intramuscular injection of toxoid[5,11,16-22,27,29] have been

TABLE 4—The Relationship Between the Blood Alpha Antitoxin Titers and the Number of Quarters Infected with Toxigenic Staphylococci

No. of quarters infected	No. of cows grouped according to titers in I.U.*			Total No. of cows	Av. titer (I.U.)
	<1	1 to 4	>4		
1	10 (9.6%)	20 (19.2%)	74 (71.2%)	104	8.2
2	2 (3.3%)	9 (15.0%)	49 (81.7%)	60	11.2
3	0 (0%)	8 (22.2%)	28 (77.8%)	36	17.1
4	0 (0%)	4 (19.0%)	17 (181.0%)	21	20.9
Total	12	41	168	221**	

* Titers are given in international units per milliliter of serum.

** Dr. L. W. Slanetz, Department of Bacteriology, University of New Hampshire, Durham, supplied the data for 149 cows. Dr. S. J. Edwards, Agricultural Research Council, Field Station, Compton, Berks, England, supplied the data for 38 cows.

found to cause an increase in the blood antitoxin titers. The addition of killed suspensions of staphylococci,[19,20] staphylococci and streptococci,[19] colibacilli,[20] 2 per cent potassium alum,[20-22] or 10 per cent aluminum hydroxide [18] to the toxoid stimulated higher antitoxin titers than did toxoid alone. The best antitoxin response occurred when alum or aluminum hydroxide was added to the toxoid.

The antitoxin response also varied with individual animals regardless of the immunizing agent. This variation has been attributed to the presence of natural antitoxin in some animals as a result of previous staphylococcic infections.[16,19] This theory is supported by the fact that young animals have lower antitoxin titers than older animals (tables 1, 2) and also have the same relationship after the injection of toxoid. The average *alpha* antitoxin titers found by Ramon *et al.*[16] after toxoid injection were 5.0 I.U. for cattle 5 to 8 months old, 11.0 I.U. for those 12 to 20 months old, and 14.0 I.U. for those 2 years old or more. They also reported that the average antitoxin titer in 9 animals that had no natural antitoxin was only 2.5 I.U. after the injection of toxoid. In addition, calves born to cows with blood antitoxin titers developed blood titers, but treatment of these calves with three injections of toxoid gave poor results. When tested seven to 36 days after the last injection, the titers in only 3 calves increased; whereas, in the other 11 calves, the titers remained the same, decreased, or disappeared.

Reports on the use of toxoid for the control of staphylococcic mastitis are conflicting. Gwatkin [5] and Minett [11] found there was no elimination of staphylococci from infected udders as a result of treatment with toxoid; Richou and Holstein [21] reported a more rapid cure of acute mastitis, an improvement in the quality of milk in cows with chronic mastitis, and the disappearance of staphylococci from the milk in 16 of 22 cows. In a later review article, they stated that of 60 cows with staphylococcic mastitis treated with toxoid, 45 were rapidly healed and 2 were improved; 13 did not benefit and this was attributed in some instances to mixed infections.[23] Recent work by Slanetz *et al.*[27] indicates that the use of toxoid or a toxoid-bacterin may be of value in preventing staphylococcic udder infections and in reducing the number of acute attacks in infected cows, but not in eliminating existing infections. Pearson [14] also used a bacterin-toxoid in one herd and reported favorable results for 21 months. The clinical mastitis and the isolations of staphylococci from milk samples were less in the vaccinated animals as compared to the control animals. During the last nine months of the experiment, however, no significant difference was found between cows of the two groups. The reinforcing doses of vaccine given every six months were considered insufficient to maintain immunity.

The work of Minett [12] with ewes would indicate that the use of toxoid is of value in reducing the severity of experimental staphylococcic mastitis. The work of Spencer *et al.*[29] and Slanetz *et al.*[27] also reported less udder reaction following intramammary challenge with cultures of staphylococci in cattle previously injected with a toxoid-bacterin. Chronic infections did develop, however, in nine of the 16 quarters of vaccinated cows studied by Spencer *et al.*[29] In the vaccinated cows studied by Slanetz *et al.*,[27] the experimental infections were mild and of short duration; whereas, in the nonvaccinated cows, the reactions were severe and generally developed into chronic infections.

DISCUSSION

Value of Antitoxins.—In reviewing the data on staphylococcic antitoxins in dairy cattle, three questions arise as to the value of antitoxins in (1) the prevention of staphylococcic udder infections, (2) the prevention of clinical mastitis, and (3) the elimination of existing infections.

The Prevention of Staphylococcic Udder Infections.—The value of staphylococcic antitoxins lies in their ability to neutralize specific toxins. It is difficult to understand how the neutralization of toxins would be of any value in preventing infections, since we do not know if toxigenic staphylococci produce toxins in milk or if toxins are essential for infection of the udder. Minett [11] found no evidence of toxin in whey prepared from milk of chronically infected quarters, although any toxin produced in chronic infections would probably be at a low level and readily neutralized by any antitoxin in the milk. Thus, any negative test for toxin would not necessarily indicate its absence. However, toxin was demonstrated in the secretions of 3 cows with gangrenous mastitis.[11] Even

if toxin were produced in milk, would it help the organism to enter the udder and successfully multiply? If we assume that nontoxigenic and toxigenic staphylococci are similarly able to grow in milk, then the prevalence of nontoxigenic staphylococcic infections indicates that toxins are not essential for establishing infection in the ducts or alveoli and that antitoxins would be of no value in preventing infection. Possibly, the toxin may be essential for invasion of the interalveolar tissue, in which case antitoxin may help prevent such invasion. Also, the blood antitoxin level may be more important than the mere presence of antitoxin and a definite amount of antitoxin must be in the milk before it can assist in preventing infection or acute attacks. This premise is supported by the higher antitoxin titers obtained after vaccination with toxoids and the apparently good results in preventing udder infections. However, evidence is contradictory; it has been shown that when cows have staphylococcic infection in more than one quarter, the antitoxin titers tend to become higher as the number of infected quarters increases. In addition, higher milk titers are found in cows with higher blood titers.

From available evidence we might conclude, therefore, that antitoxins probably have little or no value in preventing udder infection in cows that develop antitoxins as a result of natural infections. More information is needed to determine the reason for the apparently greater protection provided by injection of toxoid.

The Prevention of Clinical Mastitis.—Antitoxins apparently have some value in preventing clinical mastitis. The stimulation of staphylococcic antitoxins with toxoid reduces the severity of experimental infections and the incidence of acute attacks in natural infections. Probably, such reduction is due to the prevention of local tissue damage by the neutralization of the staphylococcic toxins. Nevertheless, if antitoxins are of value, why does a high incidence of clinical mastitis occur in chronically infected cows shortly after parturition [10,13] when the antitoxin titers of colostrum are high? What factors in colostrum or the udder apparently prevent the antitoxin from reaching the same potential of preventing attacks that seems to show during lactation?

The Elimination of Existing Infections.—There is little evidence that the antitoxins in the blood or milk have any value in eliminating existing staphylococcic infections. In fact, the whey titers are generally higher in infected quarters, and viable staphylococci have been isolated from the quarters of a vaccinated cow that had whey antitoxin titers of 12 to 51 I.U.[29]

Value of Vaccines.—Reports on vaccines for mastitis control show that the protection obtained against udder infection or clinical mastitis is incomplete. Pearson [14] also indicates that the protective value might decrease with time, even though periodic injections of vaccine are given to restimulate antibody production. Such a decrease may indicate that a high antibody titer is needed to increase resistance, which can be obtained initially only by vaccination but cannot be maintained by repeated injections of vaccine. It is not unusual, however, to find nonvaccinated cows with antitoxin titers higher than those in vaccinated cows. Therefore, another explanation may be that certain levels of antitoxin (x) and agglutinins (y) increase the resistance of the cow to udder infection, but they do not confer a complete immunity. These values, as measured by antibody titers, would vary for different animals. The use of a toxoid or bacterin alone might give as much, but no more, protection than if both were used together. The antibody levels in the heifers vaccinated with a toxoid-bacterin would, in most instances, rise to the xy level or higher. Any level higher than xy would not confer any additional resistance to udder infection with staphylococci. Animals which reached the xy level would still be susceptible to udder infection, but to a lesser degree than before. The xy level in some nonvaccinated animals would occur in time as a result of staphylococcic infections in the udder or other sites. The animals which developed the xy level of antibody, whether vaccinated or not, would have a similar degree of resistance to infection or development of clinical mastitis if infected. Vaccination should, therefore, be most valuable in heifers, because older cows would have a greater chance for developing staphylococcic infection and, consequently, a greater number would have the xy level of antibody. Actually, the xy factor may not involve any specific level of antibody; it

may be that animals will produce antibodies more quickly after an initial exposure to an antigen.

The reports that a toxoid or a toxoid-bacterin gives some protection against staphylococcic mastitis may be valid, but some of the experimental methods should be questioned for the following reasons. (1) Controls were not used by some investigators.[2,21] (2) Slanetz *et al.*[27] used controls; however, in one herd, 30 of the 39 nonvaccinated cows were not housed in the same barn with the 31 vaccinated cows. Although they were milked in the same milking parlor and were maintained under similar management, another variable was introduced into the experiment. (3) Composite milk samples from four quarters[2] or a single quarter sample[21] were used instead of separate quarter samples to determine the state of udder infection. (4) Infection was diagnosed from stained smears of incubated milk[2] instead of by bacteriologic isolation and identification of the organisms. (5) It was not indicated if the study involved only hemolytic coagulase-positive staphylococci and not other staphylococci.[21] In addition, the frequency of sampling varied among experiments. Pearson[14] pointed out that weekly or biweekly sampling is necessary if the status of infection in a herd is to be accurately determined.

Until all the factors that influence the spread of infection and the development of clinical mastitis are known, vaccination experiments on staphylococcic mastitis should not be approached in the same manner as are vaccination experiments on a contagious disease which affects the whole animal. A cow should not be classified as infected or noninfected solely on the basis of staphylococcic udder infection. For one reason, infection of a quarter with organisms other than staphylococci might prevent infection of that quarter with staphylococci. Consequently, the incidence of infections caused by other organisms should also be determined in any vaccination study. If an experiment is done in a staphylococcic-infected herd and both infected and noninfected animals are vaccinated, principal and control groups should contain comparable numbers of noninfected cows and cows infected in one, two, three, and four quarters. Frequent sampling should also be done to determine temporary infections so that this data can also be used to evaluate a vaccine. The actual value of antibodies may not be in preventing the initial invasion and growth of staphylococci in the duct system, but rather in preventing invasion of the interalveolar tissue where a more permanent type of infection can develop.

More information is needed on the role that antibodies play in the pathogenesis of staphylococcic mastitis. Because antitoxins can neutralize toxins, cows should be tested for staphylococcic antitoxins before studying the udder reaction to experimental infection or the injection of toxins. Such testing is necessary because of the fairly high incidence of antitoxins in cows free of staphylococcic udder infections. Until it is known what level of serum or whey antitoxins might significantly influence udder reaction, cows should be selected for study only if they have little or no serum antitoxin. More information also is needed on the development of staphylococcic agglutinins before the value of toxoid-bacterins for the control of staphylococcic mastitis can be assessed intelligently.

SUMMARY

A review of the literature shows that staphylococcic antitoxins are found in the blood of a large percentage of dairy cattle and that the incidence of *alpha* antitoxin is greater than *beta* antitoxin. The review of data also shows that the antitoxin titers tend to increase with the age of the animals, they are higher in cows with staphylococci-infected udders, and they tend to increase with the number of infected quarters. In addition, the *alpha* antitoxin occurs in the whey at a much lower level than in the blood. The whey titers are generally higher in the infected quarters, and the concentration appears to be correlated with an increase in the pH of the milk. The *alpha* and *beta* antitoxin titers in the colostrum may also be higher than in the blood, but they decrease rapidly after calving.

There is evidence that toxoids, which stimulate the production of antitoxins, have been used to control staphylococcic mastitis with some success. The toxoids may be valuable in preventing staphylococcic udder infections and reducing acute attacks, but they probably have little value in eliminating existing infections. Some

of the vaccination studies were questioned from the viewpoint of experimental methods, and some factors which should be considered in vaccination experiments and experimental infections were discussed.

ADDENDUM

J. B. Derbyshire (Studies in Immunity to Experimental Staphylococcal Mastitis in the Goat and Cow. *J. Comp. Path. & Therap.*, 70, (1960) : 222-231) studied the antibody response in goats to various staphylococcic vaccines and compared the degree of protection which the vaccines conferred against mastitis produced experimentally by a homologous strain of Staphylococcus. A formalized cell-toxoid with aluminum hydroxide gel stimulated serum staphylococcic antibody levels that were higher and persisted longer than those stimulated by formalized cell-toxoid, cells alone, toxoid alone, or a formalized whole broth culture. Only those vaccines that contained toxoid produced immunity against experimental staphylococcic mastitis.

The cell-toxoid with adjuvant conferred a high level of protection in goats and cows against challenge by the intramammary inoculation with living staphylococci. Only a mild, transient mastitis developed in the vaccinated goats and cows; whereas, gangrenous mastitis developed in the nonvaccinated goats and a severe, purulent mastitis with complete loss of milk secretion in the nonvaccinated cows. The duration of immunity was not determined, since all animals were challenged by an intramammary inoculation with staphylococci one week after the last injection of vaccine. The animals in these experiments were vaccinated and challenged with the same strain of Staphylococcus, and it was pointed out that work in progress suggests that the immunity against a heterologous strain might not be as effective as that against a homologous strain.

References

[1] Brown, R. W.: Unpublished data, ARS, USDA, Beltsville, Md., 1959.

[2] Collinson, R. W.: Staphylococcic Mastitis. J.A.V.M.A., 114, (March, 1949) : 147-150.

[3] Edwards, S. J., and Smith, G. S.: Staphylococcal Antitoxin in the Blood and Milk of Cattle. J. Comp. Path. & Therap., 69, (1959) : 208–215.

[4] Edwards, S. J.; Agric. Res. Council, Compton, Berks, England: Personal communication (1959).

[5] Gwatkin, R.: Further Observations on Staphylococcic Infections of the Bovine Udder. Canad. J. Pub. Health, 28, (1937) : 185–191.

[6] Kerr, W. R., Pearson, J. K. L., and Rankin, J. E. F.: The Bovine Udder and Its Agglutinins. British Vet. J., 115, (1959) : 105–119.

[7] Lasmanis, J., and Spencer, G. R.: Antibodies for Hemolysins of *Micrococcus pyogenes* in Whey and Serum of Dairy Cattle. Am. J. Vet. Res., 15, (Oct., 1954) : 517–519.

[8] Miller, W. T., and Heishman, J. O.: Staphylococcal Antitoxin in the Blood, Milk, and Colostrum of Cows. Am. J. Vet. Res., 4, (July, 1943) : 265–269.

[9] Minett, F. C.: Staphylococci from Animals with Particular Reference to Toxin Production. J. Path. &. Bact., 42, (1936) : 247–263.

[10] Minett, F. C.: Studies on Bovine Mastitis. XII. Mastitis Due to Staphylococci. J. Comp. Path. & Therap., 50, (1937) : 101-121.

[11] Minett, F. C.: Staphylococcus Antitoxin in the Blood and Milk of Cows and Other Animals. J. Comp. Path. & Therap., 50, (1937) : 173–190.

[12] Minett, F. C.: Prevention of Ovine Mastitis by the Use of Staphylococcus Toxoid. J. Comp. Path & Therap., 52, (1939) : 167–182.

[13] Neave, F. K., Dodd, F. H., and Henriques, E.: Udder Infections in the Dry Period. J. Dai. Res., 17, (1950) : 37–49.

[14] Pearson, J. K. L.: Autogenous Toxoid Vaccine in the Prophylaxis of Staphylococcal Mastitis in Cattle. J. Dai. Res., 26, (1959) : 9–16.

[15] Ramon, G., Richou, R., and Descazeaux, J.: L'antitoxine staphylococcique d'origine naturelle, chez l'homme et chez differentes espèces animales. Rev. immunol., 1, (1935) : 401–414.

[16] Ramon, G., Richou, R., Julienne, P., Jacquet, J., and Gerbeaux, C.: Le développement de l'antitoxine staphylococcique ches les bovides soumis aux injections d'anatoxine spécifique. Rev. immunol., 15, (1951) : 321–335.

[17] Ramon, G., Richou, R., Gerbeaux, C., Jacquet, J., and Julienne, P.: Sur la production simultanee, chez les bovides, des antitoxines staphylococciques *alpha* et *beta*. Compt. rend. Acad. Sci., 234, (1952) : 28–29.

[18] Ramon, G., Richou, R., Delauney, G., and Gerbeaux, C.: Sur la production, chez les bovides, de l'antitoxine staphylococcique *alpha* au moyen de l'anatoxine spécifique seule, ou additionnée d'hydroxyde d'aluminum. Compt. rend. Acad. Sci., 234, (1952) : 1657–1659.

[19] Ramon, G., Richou, R., Julienne, P., Jacquet, J., Gerbeaux, C., and Leneveu, G.: De l'influence des suspensions de streptocoques et de staphylocoques tués (anavaccin) sur l'immunité engendree, ches les bovidés, par l'anatoxine staphylococcique. Bull. Off. internat. Épizoot., 37, (1952) : 514–529.

[20] Ramon, G., Richou, R., Julienne, P., and Jacquet, J.: De l'influence comparee des suspensions de staphylocoques et de colibacilles tues (anavaccins) et de l'alun staphylococcique. Bull. Off. internat. Épizoot., 39, (1953) : 610–618.

[21] Richou, R., and Holstein, G.: Contribution a l'etude des mammites de la vache et, en particulier, de la mammite staphylococcique. Rec. méd. vét., 117, (1941) : 329–338.

[22] Richou, R., Holstein, G., and Renauldon, J.: Le développement de l'antitoxine spécifique chez les bovides soumis aux injections de'anatoxine staphylococcique seule ou additionnée d'alun de potassium. Compt. rend. Soc. Biol., 135, (1941) : 789–791.

[23] Richou, R., and Holstein, G.: L'anatoxitherapie staphylococcique en médecine vétérinaire. Results d'ensemble. Bull. Acad. vét. France, 17, (1944) : 1–13.

[24] Richou, R., Jacquet, J., Julienne, P., and Gerbeaux, C.: Sur la présence simultanée des antitoxines staphylococciques α et β d'origine naturelle dans le serum des bovides. Rev. path. comp. et hyg. gén., 52, (1952) : 231–240.

[25] Richou, R., Julienne, J., Jacquet, J., and Gerbeaux, C.: Sur la présence chez les bovides, des antitoxines staphylococciques α et β, d'origine naturelle. Compt. rend. Soc. Biol., 146, (1952) : 177–179.

[26] Richou, R., Gerbeaux, C., Jacquet, J., and Julienne, P.: Rapport entre l'immunité antistaphylococcique d'origine naturelle et l'infection de la mamelle chez les femelles bovines. Compt. rend. Soc. Biol., 146, (1952) : 636–638.

[27] Slanetz, L. W., Bartley, C. H., and Allen, F. E.: The Immunization of Dairy Cattle Against Staphylococcal Mastitis. J.A.V.M.A., 134, (1959) : 155–161.

[28] Slanetz, L. W.; University of New Hampshire, Durham: Personal communication (1959).

[29] Spencer, G. R., Stewart, J. H., and Lasmanis, J.: Preliminary Report on Immunization of Animals Against *Micrococcus pyogenes*. Am. J. Vet. Res., 17, (Oct., 1956): 594–598.

[30] Taylor, M. E.: Antibody Production in the Bovine Mammary Gland. M.S. Thesis, Iowa State University Library, Ames, 1957.

[31] Thörne, H.: The Antistaphylolysin Titre in Cattle, with Special Reference to Chronic Staphylococcal Mastitis. Nord. Vet.-med., 10, (1958): 63–75.

SUMMARIO IN INTERLINGUA

Antitoxinas Staphylococcic in Bestial de Lacte. I.A. Revista del Litteratura

Un revista del litteratura indica que antitoxinas staphylococcic es incontrate in le sanguine de un grande procentage de bestial de lacte e que le incidentia de antitoxina *alpha* es plus que illo de antitoxina *beta*. Le revista del datos etiam monstra que le titros de antitoxina tende a montar con le etate del animales, que illos es plus alte in vaccas con uberes inficite per staphylococcos, e que illos tende a montar con le numero del inficite quartos de ubere. In plus, antitoxina *alpha* ha multo plus basse titros in le sero de lacte que in le sanguine. Le titros in le sero de lacte es generalmente plus alte in le caso de lacte ab quartos inficite. Il pare que le concentration del antitoxina es correlationate con un augmento del pH del lacte. Le titros pro antitoxina *alpha* e *beta* in le colostro es etiam possibilemente plus alte que in le sanguine, sed illos declina rapidemente post le parturition.

Il existe constatattiones a suggerer que toxoides que stimula le production de antitoxinas ha essite usate con un certe grado de bon successo in le lucta contra mastitis staphylococcic. Le toxoides es forsan de valor in le prevention de infectiones staphylococcic del uberes e in reducer le incidentia de attaccos acute, sed il es probabile que illos non contribue multo al elimination de infectiones jam existente. Certe studios del problema de vaccination esseva questionate ab le puncto de vista del methodologia experimental. Es discutite certe factores que debe esser prendite in consideration in experimentos vaccinational e in infectiones experimental.

Bovine Malignant Catarrhal Fever in Michigan. II. Pathology

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FEW REPORTS on the pathology of bovine malignant catarrhal fever (MCF) have been published in the United States. Smith and Jones,[10] in their recent book, "Veterinary Pathology," state that "considerably more study of malignant catarrh is needed, as are better methods for its recognition." The few available reports of the disease in North America are mostly of a clinical nature or have dealt with only the gross lesions.[2,3,6,7] Round and mononuclear cell infiltrations in the brain, liver, and kidneys were reported from Canada.[9] A report from Ohio describes a serocatarrhal to diphtheritic inflammation involving the mucous membranes; cytoplasmic inclusions were reported in the epithelial cells of the mucous membranes.[4] It is hoped that the studies reported herein will contribute to the knowledge regarding the nature and diagnosis of this disease, particularly the recognition of relatively atypical cases which, in our experience, were quite common.

MATERIALS AND METHODS

Necropsies were conducted on 31 animals with MCF. Tissues were fixed in 10 per cent formalin with sodium acetate.[1] The tissues were dehydrated in a series of graded alcohols, cleared in xylene, and embedded in paraffin. Sections were cut at 6 to 7 μ and stained with hematoxylin and eosin. Sudan IV was used to stain for fat.[5] Other special stains used were Lendrum's, Pollak's trichrome, Masson's trichrome, Verhoeff's elastica stain, Mallory's aniline blue collagen stain, and the phloxine-methylene blue stain. These stains were used according to formulas and methods in the "Manual of Histologic and Special Staining Technics."[1] The phloxine-methylene blue stain was modified; the slides were stained in phloxine for three hours at 56 C. or overnight, followed by decolorization in 1 per cent acid alcohol for ten seconds.

PATHOLOGIC FINDINGS

SKIN AND MUCOUS MEMBRANE

The animals presented for necropsy were usually markedly under-weight. The hair was dull and roughened; occasionally, tufting of the hair was found (in chronic cases hairless areas were present), particularly in the cervical area. In many cases, thickened crusts (1 to 2 mm. in diameter) were observed on the skin, most often in the cervical region, but occasionally scattered over the entire body. The vulva was often markedly reddened. In many instances, marked lesions of the udder were found: cracking and crusting of the skin of the teats, areas of erythema, and raised papules, 1 to 2 mm. in diameter. Lesions of the muzzle varied from thickening and scaling to complete erosion of the muzzle epithelium, exposing a raw, reddened surface. Generally, there was a profuse mucopurulent nasal discharge.

The microscopic lesions of the skin, as well as other stratified squamous epithelia, have been grouped for convenience of description. Representative sections from the muzzle, vulva, udder, eyelids, affected skin areas, and squamous portions of the digestive tract were examined, as were specimens from macroscopically normal areas. Many of the apparently normal tissues contained excellent examples of microscopic lesions. By comparing tissues from these areas with tissues from more severely affected sites, the pathogenesis of the lesions could be reconstructed. Lesions in all affected animals were remarkably similar, although their distribution varied from animal to animal *i.e.*, the variations were quantitative rather than qualitative.

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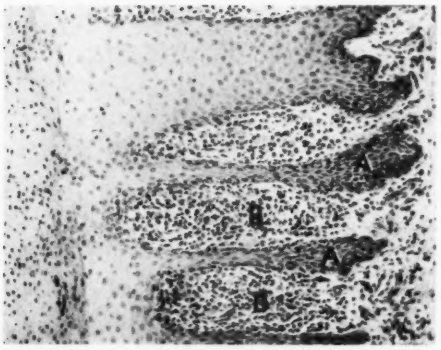

Fig. 1—Early lesion of tongue with clubbing of the rete pegs due to hyperplasia (A) and cell accumulations in the papillae (B). H & E stain; x 90.

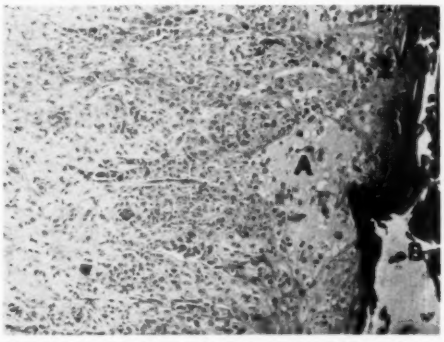

Fig. 2—Vesiculation (A) and scaling of hyperkeratinized layer (B) in epithelium of the teat. H & E stain; x 90.

The process appeared to begin as a multiplication of cells of the basal layer of the epithelium, together with an increase of connective tissue cells and lymphocytes in the papillae (fig. 1). Increased cellular activity was apparent around blood vessels which were markedly congested. The epithelial cells formed loose networks which were interconnected by fine cytoplasmic strands. Occasional ballooning cells with condensed acidophilic cytoplasm were found in the central areas of the rete pegs. Many of the rete pegs were elongated and club-shaped. The basal cell layer was not distinct, due to the loss of polarity of the nuclei and proliferation of the cells (mitotic figures were frequently observed in the basal area). Occasional necrotic epithelial cells, identified by their bright pink cytoplasm and fragmented nuclei, were scattered through the cellular masses in the dermal papillae. Thickened keratin layers often maintained the external continuity of the tissue; in other instances, portions of the keratin layer were elevated by the underlying disease processes. In more severe lesions, large clumps of ballooning cells underwent reticulating colliquation with resultant vesicle formation in the upper epithelium (fig. 2). The vesicles contained cellular debris, protein-containing fluid and, occasionally, abundant polymorphonuclear leukocytes. Occasionally, Russell's bodies were observed in the epithelial cells. The hypercellular masses in the dermis were often pleomorphic. Many spindle-shaped connective tissue cells with large pale, oval nuclei (fibroblasts) were present. Numerous lymphocytes, many of which were large immature forms, were seen often. Small numbers of eosinophils, macrophages, plasma cells, and mast cells were usually present. These changes, not restricted to the upper dermis, were evident around blood vessels in the deeper portions of the dermis. Hyperplasia in and about vessels and capillaries was usually pronounced and, in some instances, obstruction of the vessels occurred due to masses of endothelial cells which projected into the lumina (fig. 3). Hyperplasia of the epithelial cells of the skin adnexa was often prominent. Occasional lymphocytes and proliferating endothelial cells were observed in these areas. Vessels in the underlying muscle were sometimes involved. Generally, it may be said that in early lesions hyperplasia was paramount; in the more advanced processes, infiltration of

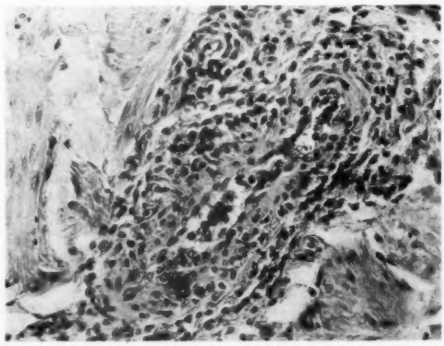

Fig. 3—Artery in dermis of teat with endothelial (A) medial (B) and adventitial proliferation (C). Area of proliferation about a capillary (D). H & E stain; x 167.

lymphocytes and sometimes eosinophils was prominent. Special stains were used to identify the proliferating tissue in the dermis and about the blood vessels. Mallory's aniline blue technique, Verhoeff's elastic tissue stain, Van Gieson's collagen stain, and Masson's and Pollak's trichrome stains indicated that this was collagenous connective tissue.

EYES

Bilateral corneal opacity was usually marked; however, in some instances, slight or no cloudiness was encountered. The conjunctival vessels were generally injected. The eyelids were thickened and rounded. Abundant mucopurulent exudate was often present in the inner canthi.

The corneal opacity observed macroscopically and the nature of its development (initial peripheral cloudiness which gradually spread to the center of the cornea) may be explained by the lesions found on microscopic examination. The most severe changes were found in the area of the corneoscleral junction, but extended into the cornea, becoming less marked as they approached the center of the cornea. Essentially, the pathologic process was proliferative and infiltrative. In the conjunctival epithelium, ballooning degeneration and irregular hyperplastic projections into the lamina propria were usually seen. In these areas, mitotic activity was evident. In some areas, the epithelium was absent. The underlying lamina propria and sclera, particularly in the anterior portions, contained abundant, pleomorphic, cellular aggregations composed of mononuclear cells and lymphocytes, and some eosinophils and plasma cells. Large connective tissue cells were increased in number (fig. 4). Many mitotic figures were observed in large cells of varying shapes which were difficult to classify. Swelling, rounding, and hyperplasia of capillary endothelial cells were observed. Blood vessels were affected by proliferative and infiltrative lesions as previously described. In the corneal epithelium, ballooning degeneration and occasional areas of erosion were seen. Rupture of the substantia propria of the cornea sometimes occurred. The connective tissue in the substantia propria of the cornea was often granular and somewhat disrupted. Polymorphonuclear leukocytes were occasionally abundant in the cornea; the humors of the eye sometimes contained polymorphonuclear leukocytes as well as monocytes, lymphocytes, and cellular debris. Cell accumulations, similar to those described in the conjunctival lamina propria and sclera, were found in the iris, ciliary body, and retina. Occasional vascular lesions occurred in the connective tissue of the optic nerve, periorbital fat, and orbital muscles. Congestion of seemingly all parts of the eye was marked.

DIGESTIVE TRACT

Mouth, Pharynx, and Esophagus.—The mucous membranes of the mouth usually showed severe necrotizing processes which varied from distinct ulcers of irregular shape and size to diffuse necrosis of the epithelium of almost the entire cavity, in which the epithelium was easily scraped away with a knife. Commonly, a thick, grayish white, diphtheritic membrane was found in the pharynx; it often involved the esophagus and trachea also. This diphtheritic membrane covered a necrotic mucous membrane. When distinct ulcers were present, they might be found in any area of the oral cavity; usually, the remaining intact membrane was dull and gray. In some cases, petechial hemorrhages were present on the ventral surface of the tip of the tongue. The diphtheritic inflammation in the pharynx usually extended into the proximal portion of the esophagus for a short distance. Occasionally, small ulcers or diffuse necrosis was found throughout the length of the esophagus. Flattening of the longitudinal folds and dullness of the

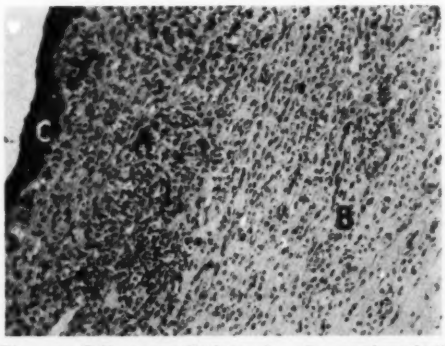

Fig. 4—Cell accumulations in the conjunctival lamina propria (A) and sclera (B) near the limbus. Notice the hyperplasia of the conjunctival epithelium (C). H & E stain; x 90.

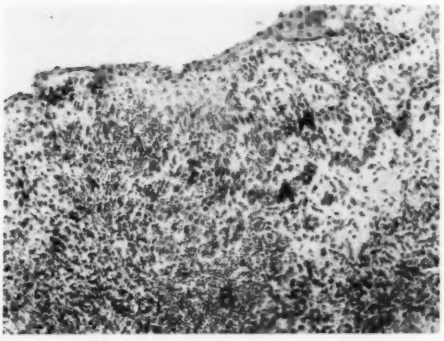

Fig. 5—Epithelial hyperplasia (A) and hemorrhage (B) in papilla of rumen. H & E stain; x 90.

esophageal epithelium were observed in some cases.

Microscopically, the stratified squamous portions of the digestive tract contained lesions as previously described. Blood vessels were markedly congested and the lesions resembled those described for the skin. The salivary glands were often the site of proliferative and infiltrative processes. The epithelial cells of the salivary ducts were often hyperplastic and disarranged; proliferation and infiltration of cells were observed in the laminae propriae of the ducts. The secretory units adjacent to the affected areas were compressed.

Rumen and Recticulum.—Gross lesions were variable in the rumen and recticulum. Occasionally, diffuse areas of hemorrhage or erosion, or both, were found. In a few cases, the papillae of the rumen showed particularly striking hemorrhages. Microscopically, the lesions involved the squamous epithelia (fig. 5) as previously described for skin.

Abomasum.—The abomasum was commonly the site of extreme congestion, hemorrhage, and ulcers up to 3 cm. in diameter (fig. 6). Microscopically, scattered areas of epithelial desquamation were observed in many cases. The primary change appeared to involve the blood vessels, particularly those in the submucosa. These vessels were surrounded by cuffs of lymphocytes and monocytes, and occasional eosinophils and plasma cells. Adventitial and endothelial cells were often increased in number; the media often contained areas of fibrinoid degeneration, pyknotic nuclei, or foci of proliferating cells which appeared to extend from the adventitia or endothelium. Vascular congestion was generalized. Occasionally, hemorrhages were seen in the mucosa.

Small Intestine.—The small intestine was rarely involved severely. Abundant mucus was usually present. Rarely, petechiae, congestion, or small erosions were found. Microscopically, the lesions resembled those of the abomasum, except that they were found less frequently.

Large Intestine.—In a few instances, the ileocecal valve was markedly hemorrhagic, thickened, and edematous (fig. 7). In several cases, the cecum and colon were congested and petechial; they sometimes contained small erosions. Similar changes were seen in the rectum; petechial hemorrhages were usually arranged linearly on the crests of the rectal folds.

Microscopically, severe lesions were found in the large intestines of several animals.

Fig. 6—Circumscribed (A) and linear (B) erosions in the abomasum in an animal with acute malignant catarrhal fever.

Fig. 7—Diffuse hemoorrhage and edema in the ileocecal valve (arrow) and adjacent area in an animal with acute malignant catarrhal fever.

Hemorrhage into the mucosa of the ileocecal valve was common; smaller hemorrhages were observed in the mucosae of the colon and rectum. Minor desquamation of the epithelium was seen in scattered areas throughout the large intestine. In several cattle, blood vessels, particularly in the submucosa, were surrounded by cuffs of lymphocytes, monocytes, eosinophils and plasma cells. Adventitial and endothelial cell proliferation was usually evident in such cases and often extended into the media. Submucosal edema was generally pronounced in the affected portions of the large intestine.

Liver.—Prominent gross lesions were not found in the liver. Usually, swelling and indications of fatty changes were present. Microscopically, conspicuous lesions were consistently found in the periportal areas. These areas were greatly distended by cell accumulations which compressed, but did not extend between, the hepatic cords (fig. 8). Many of the liver cells which were so compressed by the cell accumulations showed pyknosis or karyorrhexis. The periportal cell collections were composed of several types of cells, but monocytes and lymphocytes were the most prominent (many were markedly pleomorphic). More variably found were eosinophils, plasma cells, and, occasionally, polymorphonuclear leukocytes. Mitotic activity in these areas was pronounced. The blood vessel walls were often greatly masked by the collections of cells, being recognized only by a central collection of erythrocytes surrounded by an occasional area of recognizable mural structure. The bile duct epithelium was often hyperplastic, as evidenced by an irregular piling up of pleomorphic duct cells. Capillaries in the portal area were markedly congested and the endothelial cells were enlarged and hyperplastic. Sections from 4 of the 31 affected animals were stained for fat. Mild to severe diffusely distributed fatty changes of the hepatic cells were seen.

Gallbladder.—In a few cattle, there was macroscopic evidence of congestion and thickening of the wall of the gallbladder. Microscopically, the changes followed the pattern found in other organs. The epithelium was intact except for an occasional small area. There was an increase of primarily monocytes, fibroblasts, and lymphocytes in the lamina propria.

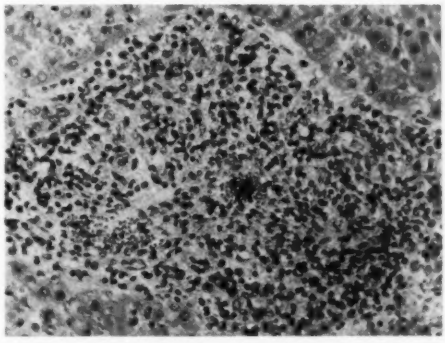

Fig. 8—Cell accumulation limited to the periportal area of the liver. Lumen of vessel with wall is masked by accumulation of cells (A). H & E stain; x 167.

Plasma cells and eosinophils were observed in small numbers. A proliferative reaction was observed around the blood vessels, particularly the capillaries. Occasionally, glands appeared hyperplastic in the areas of proliferative activity in the lamina propria. The muscle layers were disrupted by cellular infiltrates which appeared to emanate from blood vessels affected by the proliferative and infiltrative processes.

Pancreas.—Macroscopic lesions of the pancreas were not observed. Microscopic lesions were not prominent. Mild proliferative activity among fibroblasts was present about occasional ducts; some of the duct cells were somewhat disoriented, suggesting the possibility of an early hyperplastic change. The capillaries were moderately congested and sometimes showed mild hyperplasia of the endothelial cells.

LYMPHATIC SYSTEM

Lymph Nodes.—The lymph nodes of the head were usually enlarged, edematous, and congested. In many instances, the lymph nodes throughout the body were similarly affected and always contained microscopic lesions. The changes were consistent and are considered to be of diagnostic value. Blood vessels in the capsule, pulp, and perinodal fat were often markedly affected by proliferation of adventitial and endothelial cells, as well as by accumulations of lymphocytes, monocytes, plasma cells, and eosinophils. The cell accumulations extended into the capsule and trabeculae from the vessels, masking the normal structure (fig. 9). In some cases, the follicular

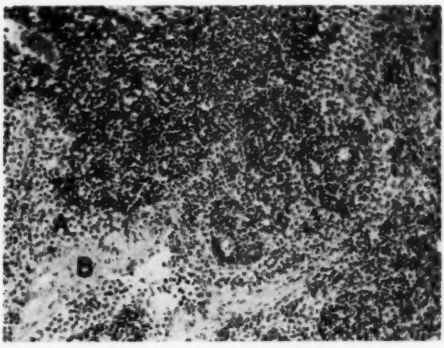

Fig. 9—Lymph node showing reticulum cell hyperplasia (A), degeneration of connective tissue in a trabecula (B), and infiltration of a trabecula (C). H & E stain; x 90.

architecture was absent or represented by a few lymphocytes; in other cases, the follicles were hyperplastic. In the majority of cases, there was marked reticular cell proliferation, particularly along the trabeculae. Numerous mitotic figures were observed. Marked congestion and edema were frequently found; rarely, hemorrhage was present. The parenchyma often bulged against the capsule, in which case, clusters of pyknotic lymphocytes were observed along the capsule. Eosinophils, plasma cells, and polymorphonuclear leukocytes were variably found in the pulp.

Spleen—The spleen showed no remarkable gross lesions. Microscopic lesions, similar to those in the lymph nodes, were seen in some instances. Trabecular disruption and hyalinization were evident adjacent to involved vessels. Follicular hyperplasia was often evident. Large amounts of hemosiderin were present in some cases.

CARDIOVASCULAR SYSTEM

Heart.—Hemorrhages were frequently observed on the epicardium. The microscopic lesions followed the pattern observed in other organs. Perivascular cuffs of adventitial cells, lymphocytes, and occasional monocytes, and, sometimes, minor infiltrations of eosinophils, plasma cells, and polymorphonuclear leukocytes were present. Both fibrinoid degeneration and accumulations of proliferating cells were observed in the media. Proliferation of endothelial cells was seen in some vessels. The lesions occasionally impinged on adjacent muscle fibers but usually extended along the connective tissue tracts from the blood vessels. Mild to severe fatty degeneration was observed in specially stained cardiac muscle fibers from 4 animals. Vascular lesions were not present in the hearts of all affected animals, and not all vessels in an affected section had lesions.

Aorta.—In 1 animal, papular elevations, 1 to 2 mm. in diameter, were seen on the intimal surface of the aorta. These proved, microscopically, to be focal areas of endothelial proliferation. Whether this should be considered an incidental finding or a part of the MCF syndrome cannot be decided on the basis of one specimen, although the nature of the lesion suggests that it was a part of the disease process. Accumulations of cells in and about the vasa vasorum of the aorta, similar to those described for the heart, were occasionally observed.

RESPIRATORY SYSTEM

Nasal Passages and Trachea.—Commonly, a thick, greenish, fetid, fibrinonecrotic membrane covered the congested nasal mucous membranes (fig. 10). Usually the proximal portion and occasionally the entire length of the trachea contained a similar diphtheritic layer. Scattered petechial hemorrhages were observed in both the nasal passages and trachea. The epithelial surface of the trachea was generally dull and yellowish in appearance.

Microscopically, the epithelium of the nasal passages and trachea contained many ballooned cells. Fibrinonecrotic exudate was piled up on the surface. In several cattle, little or no recognizable epithelium remained. Aggregations of lymphocytes

Fig. 10—Diphtheric membrane (A) and hemorrhage (B) in the nasal sinus of an animal with acute malignant catarrhal fever.

and monocytes were scattered throughout the lamina propria. Connective tissue proliferation in the lamina propria was evident. Capillaries were markedly congested and the endothelial cells were swollen and hyperplastic.

Lungs.—There were pleural hemorrhages and areas of firm, red hepatization in some lungs.

In several cattle there were marked microscopic lesions in the lungs, consistent with the pathologic picture found in other organs. The larger bronchioles were the site of marked proliferative and infiltrative changes, although not all of these bronchioles were affected. The lamina propria contained cellular aggregations of monocytes, lymphocytes, and fibroblasts, and occasional eosinophils, plasma cells, and polymorphonuclear leukocytes which extended into the muscularis (fig 11). The bronchiolar epithelium was pushed into the lumen by the underlying cell collections; loss of polarity and hyperplasia of the bronchiolar epithelium were usually present in affected bronchioles. Many of the respiratory epithelial cells were ballooned. Often there was poor demarcation between the epithelium and lamina propria. The lumina of the bronchioles contained mucus, cellular debris, and polymorphonuclear leukocytes. Capillary endothelial cells were generally swollen and hyperplastic. The alveolar walls were thickened by an increased number of septal cells. The alveoli were filled with erythrocytes in many areas. There were marked adventitial and endothelial proliferations in the large and small blood vessels; fibrinoid degeneration was common in the media, although increased numbers of cells were noticed which appeared to extend from the proliferative endothelial or adventitial areas. Accumulations of monocytes and lymphocytes, and occasional eosinophils, plasma cells, and polymorphonuclear leukocytes were present in the adventitial cuffs. All of the blood vessels were extremely congested.

URINARY SYSTEM

Kidneys.—The kidneys were usually congested and often contained petechial hemorrhages. In 1 animal, white focal areas were present on the cortical surface.

The microscopic changes in the kidney affected the blood vessels and Bowman's capsule. These changes were consistent with lesions in other organs and were essentially proliferative and infiltrative in nature. Blood vessels, surrounded by loose cuffs which were composed of adventitial cells, lymphocytes, monocytes, and some eosinophils and plasma cells, were scattered throughout the kidney, although not all vessels were affected. In many instances, the glomerular capsule was masked by infiltrations of lymphocytes and monocytes. These areas were most frequently seen surrounding the afferent and efferent arterioles. In view of the lesions of the vessels in the kidney, as well as in other organs, it was felt that the accumulations originated from the arterioles. The lesions were usually semilunar in distribution, but occasionally extended completely around the glomerulus. The process occasionally extended into the capillary tuft. Compression of the tubules in the area adjacent to the cell accumulations was evident. Albuminous degeneration was usually present in the tubules. Marked congestion of the intertubular capillaries and, occasionally, hemorrhage were found in both the cortex and medulla. Pathologic changes in the kidney were frequent but not constant. Mild to severe tubular and glomerular fat accumulations were revealed by special staining of sections from 4 of 31 animals.

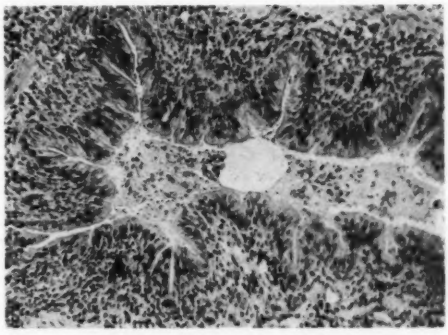

Fig. 11—Cell accumulations in the lamina propria of a bronchiole (A). H & E stain; x 167.

Ureters.—Grossly, the ureters appeared swollen and congested. Microscopically, ballooning degeneration was seen in the transitional epithelium; little or no recognizable epithelium remained in some areas. Occasional sections of epithelium appeared somewhat hyperplastic and blended with the highly cellular lamina propria. The latter contained numerous monocytes, lymphocytes, and large, pale, connective tissue cells, together with a smaller

Fig. 12—Hemorrhage and thickening of the wall of the urinary bladder in an animal with acute malignant catarrhal fever.

number of eosinophils, plasma cells, and occasional mast cells. In blood vessels in the same lamina propria and muscularis, fibrinoid degeneration of the media and accumulations of cells in the adventitia resembled those previously described for the skin. Marked congestion in most vessels was evident.

Urinary Bladder.—Macroscopically, the bladder was severely affected in several animals. The wall was greatly thickened. The mucosa was thrown into large folds; mucosal hemorrhage was often severe (fig. 12), accounting for the hematuria observed clinically in several affected animals.

Microscopically, hemorrhage was often pronounced in the submucosa. The epithelium was often absent; in other instances, transitional cells were undergoing ballooning degeneration. The lamina propria was markedly hypercellular, containing large mononuclear cells, lymphocytes, fibroblasts, and some eosinophils and plasma cells (fig. 13). In a few areas, the cellular masses in the lamina propria blended with the transitional epithelium. Blood vessels were severely involved, undergoing alterations similar to those previously described. Capillary endothelial cells were enlarged and hyperplastic. Congestion was marked. The smooth muscle often contained evident vascular lesions which produced disruption of muscle layers.

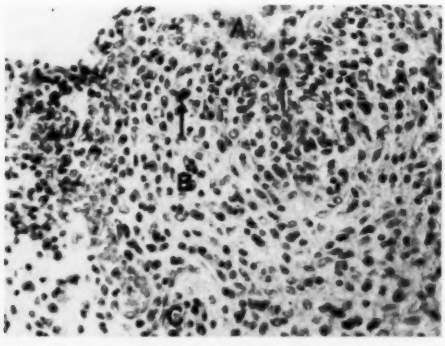

Fig. 13—Loss of transitional epithelium in the urinary bladder. Epithelial remnant (A) and cell accumulations in the lamina propria (B). Notice the mitoses (arrows). H & E stain; x 267.

REPRODUCTIVE SYSTEM

Uterus, Ovaries, and Oviducts.—The uterus, ovaries, and oviducts did not present any macroscopic lesions other than congestion.

Microscopic sections indicated that severe involvement of the system may occur. Portions of uteri examined had lesions in accord with those observed in other organs. Blood vessels throughout the uterus were affected by proliferative, infiltrative, and, sometimes, degenerative phenomena. Endothelial activity was observed in both capillaries and larger vessels. Cuffs of cells surrounded many vessels; cuffs were composed of adventitial cells and infiltrating cells as previously described. The media often was either involved with cellular accumulations or undergoing fibrinoid degeneration, or both. Fluid accumulations and cell collections were scattered throughout the uterus. Patchy areas of glandular hyperplasia appeared to contribute to the proliferative picture. Vascular lesions were also found in the ovaries. Many areas of hemorrhage were found in and about the affected vessels.

One fetus and its fetal membranes were examined. Vascular lesions were present in the chorionic villi, but none could be found in the fetus. The oviducts contained cell accumulations in the lamina propria, as well as vascular lesions.

Mammary Glands.—Specimens of mammary tissue from both lactating and nonlactating cows contained proliferative and minor infiltrative processes. Endothelial cells of small capillaries in the interstitial tissue were enlarged, rounded, and hyperplastic. Marked congestion of the blood vessels was present. In the alveolar cells in many of the secretory units, there was a loss of nuclear polarity and piling up of pleomorphic alveolar cells in a manner which

caused them to blend with the activated endothelial cells in the interstitium. In some instances, the lumina of the glands were obliterated by masses of proliferative cells. Similar changes were present in the ducts and teat cisterns. The epithelial cells appeared markedly ballooned and the lamina propria was markedly hypercellular (fig. 14). Monocytes and lymphocytes and some plasma cells, eosinophils, and mast cells were present. In some areas, the epithelium and lamina propria were poorly demarcated. Vascular changes were the same as observed in other organs. The severe lesions in the mammary gland may account in part, at least, for the sharp decline in milk production in lactating animals, a prominent clinical feature.

Testes.—Macroscopically, the testes from 1 bull were congested. Sections revealed that arteries and veins in both parenchyma and capsule were surrounded by cuffs of proliferating adventitial cells, lymphocytes, and occasional wandering monocytes. The media of many of the vessels had a fibinoid appearance; cell accumulations sometimes were present in the media. The endothelial cells in some vessels were enlarged and hyperplastic. Capillaries in the interstitial area contained enlarged and hyperplastic endothelial cells. Compression of the seminiferous tubules was evident. All of the blood vessels were engorged with blood.

Endocrine System

Thyroid.—Macroscopic lesions were not observed in the thyroid. Microscopically, there was marked hyperplasia of follicular cells which often obliterated the lumen (fig. 15). The process extended into the interstitial tissue, blending with proliferating endothelial cells. Occasional lymphocytes were present in the interstitial areas. Pyknotic follicular cells were frequently observed in the lumina of some of the affected alveoli.

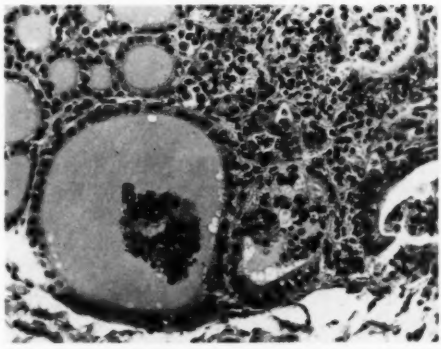

Fig. 15—Hyperplasia of follicular cells in the thyroid (A) and pyknotic cells in the lumina of the follicles (B). H & E stain; x 167.

Adrenals.—Macroscopic changes were not observed in the adrenals. Microscopic lesions of the blood vessels, consistent with those found in other organs, were commonly found in the adrenal glands in both the capsular and parenchymal areas (fig. 16). Compression and disruption of the adrenal architecture were apparent in the areas adjacent to the vascular lesions. Fibrinoid degeneration of the media and endothelial and adventitial proliferation were observed. Lymphocytes and monocytes and some eosinophils, plasma cells, and mast cells were present in the perivascular tissue extending into the capsule. Occasional areas of hemorrhage were found; blood vessels were markedly congested. Occasionally, affected vessels were present in the fat surrounding the adrenal glands.

Pituitary.—No macroscopic lesions of the pituitary gland were observed. Microscopic changes were limited to the blood vessels, particularly those in the connective tissue. Changes were similar to the vascular lesions in other organs.

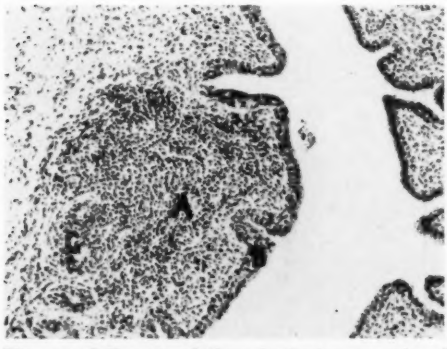

Fig. 14—Cell accumulations in lamina propria of teat cistern (A), ballooning degeneration of epithelial cells lining the cistern (B), and reaction around a duct (C). H & E stain; x 90.

Nervous System

Central Nervous System.—Gross lesions of the brain were limited to mild to marked congestion and edema of the meninges. The most prominent and consistent microscopic alterations involved the blood vessels, both

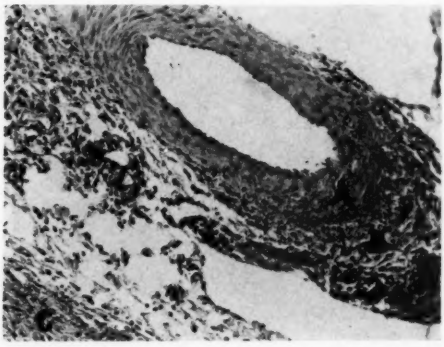

Fig. 16—Degeneration in the media (A) and cell accumulations in the adventitia (B) in a periadrenal artery. Capsule of adrenal gland (C). H & E stain; x 90.

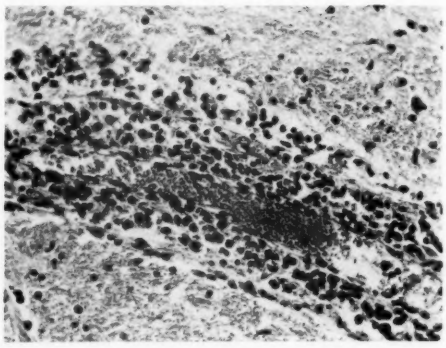

Fig. 17—Perivascular cuffing of vessel in medulla oblongata. H & E stain; x 167.

arteries and veins. The vessels were surrounded by cuffs, predominantly monocytes and lymphocytes (fig. 17). Occasionally, eosinophils were abundant. The majority of the mononuclear cells were seemingly interconnected by fine, pale, cytoplasmic strands. The lymphocytes were pleomorphic; large, young forms were abundant. Mitotic activity was frequently observed in the cuffs. In some vessels, the endothelial cells were enlarged and rounded; occasionally, they were piled up, suggesting mild hyperplasia. Free red blood cells were often present in the cuffs and perivascular spaces; they appeared to have leaked through the loose, disarranged structure of the vessel walls. Often the Virchow-Robin spaces were obliterated by the thickened walls and congestion in the vessels; encroachment upon the surrounding brain substance was apparent in many areas The capillary endothelial cells were generally enlarged and hyperplastic. Sections of the central nervous system examined included meninges, medulla, cerebellum, cerebrum, thalamic area, hippocampus, and spinal cord, all of which contained lesions. In several animals, the choroid plexus showed both infiltration of numerous mononuclear cells and proliferation of the capillary endothelial cells.

Nonspecific changes also were usually present in the brain. Marked congestion and edema were often pronounced. The neurons appeared normal in many animals and degenerative changes, when present, were not marked. However, there were varying degrees of neuronal degeneration, as evidenced by eccentricity of the nucleus, chromatolysis, and ghost cells. Satellitosis and neuronophagia were present in some animals but were usually not marked. It is believed that the neuronal changes were due to the impaired circulation and pressure produced by affected vessels rather than to specific infection of the neurons. Both generalized and focal increases in glial cells were observed.

Inclusion bodies, as described by Stenius,[11] were found in the cytoplasm of the neurons of the vagoglossopharyngeal nucleus in 73 per cent of the animals examined (fig. 18). These bodies were observed in two patterns of distribution; *viz.*, single or multiple inclusions scattered throughout the cytoplasm, or conglomerates composed of numerous spherical bodies located adjacent to the nuclear membrane. Distinct halos were often visible about the bodies. The inclusion bodies varied from 300 to 500 mμ in diameter. Both degenerated and ap-

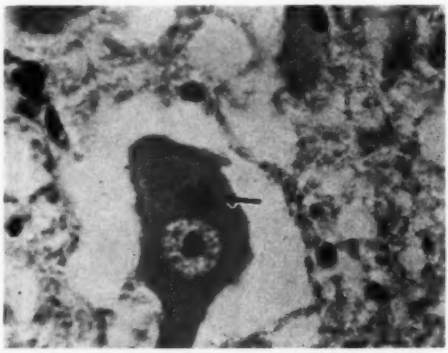

Fig. 13—Intracytoplasmic inclusion bodies (arrow) in a neuron in the medulla oblongata. Phloxine-methylene blue stain; x 263.

parently normal neurons were affected. The bodies were difficult or impossible to see with the routine hematoxylin and eosin stain. Among the stains used, Mallory's phloxine-methylene blue stain gave the best results. Not only was excellent differentiation of the bodies obtained (bright to dark red), but the Nissl substance was well stained by this method. Other stains used to demonstrate these bodies were Shorr's III, Masson's trichrome, Pollak's trichrome, and Lendrum's stains.

In an attempt to demonstrate the diagnostic importance of these striking bodies, sections of medullae from cattle routinely necropsied were examined. Similar bodies were observed in 18 of 47 animals examined. There was no evidence of the presence of malignant catarrhal fever at the time of necropsy of these animals. Therefore, we do not believe that these bodies are of specific diagnostic importance.

Gasserian Ganglia.—Gasserian ganglia were removed from several animals for examination for inclusion bodies by special staining techniques. No inclusion bodies were found, though the observations included several animals wherein such bodies were demonstrated in the medulla. However, microscopic lesions were found, consisting of the previously described proliferative and infiltrative changes of the vessel walls which extended into the connective tissue surrounding the cell bodies of the neurons. There appeared to be a mild increase in the capsular cells. Many of the nerve fibers in affected areas were fragmented.

Discussion

Grossly and microscopically, it is evident that the causative agent of MCF may produce lesions in all systems of the body and, therefore, it is pantropic in nature. Thus, it is no more appropriate to classify this disease as primarily an encephalitis than to so classify hog cholera or canine distemper. The lesions in the brain primarily affect the blood vessels; that neurons are not severely involved is indicated by microscopic examination and by the lack of extreme nervous manifestations in most animals.

The pattern of the lesions is consistent in all systems. Essentially, two different processes are involved: (1) proliferation of connective tissue, endothelial, and epithelial cells is widely distributed in epithelial tissues and blood vessels and (2) infiltration is generalized, affecting the laminae propriae of many structures, as well as the periportal area of the liver and blood vessels. We can logically attribute the necrosis which may occur, particularly in the squamous epithelia, to any one or, more likely, a combination of several causes. First, the surface integrity of structures such as the skin and mucous membranes is disrupted by the ballooning degeneration, vesiculation, and loss of epithelium which exposes the tissue to secondary invaders. Second, the involvement of the vasculature by proliferative and infiltrative processes which may obstruct the lumen or disrupt the architecture of the wall, thus affecting the circulation to the area, is certainly important. This mechanism is well illustrated in the abomasum and intestines. Here one finds primarily only a desquamation of epithelium with an involvement of vessels, particularly in the submucosa, which appears to be the logical cause. Even if not obstructed, these vessels cannot contract or dilate normally in response to the tissue demands for blood. Third, the great increase in perivascular cellular elements would seem to produce a pressure which may not only affect the cells directly but probably also shuts off lymphatic and vascular channels. In addition, the functional activity of an organ may be expected to be affected by the lesions in that organ, as well as by influences from other involved organs.

We should like to present those features of the disease which, in our experience, have proved to be of the greatest diagnostic value. One must remember that the distribution of many of the lesions is highly variable. The typical "head and eye" form is obvious; however, cases have been encountered wherein only intestinal lesions have been observed. A case is described in Canada in which only inflammation of the trachea and large bronchi and ecchymoses in the heart were found at necropsy.[9] Historically, contact with sheep and indication of a previous sporadic occurrence (which may be discovered only by further questioning of the owner) are helpful features. Clinically, MCF should be considered when there is a temperature of approximately 106 F. early in the disease, no response to antibiotics, indications of skin involvement (erythema, papulation, scabbing), corneal cloudiness, rapid weight loss, and death. Indicative postmortem findings are: diphtheritic inflammation in the nasal passages,

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oral cavity, and trachea; enlarged, edematous, and hemorrhagic lymph nodes; and hemorrhage and erosions in the digestive tract and bladder. Microscopically, the most diagnostic lesions are the constant cellular accumulations (primarily monocytes and lymphocytes) around the blood vessels in the brain and in the periportal areas of the liver. The microscopic lesions in the skin, lymph nodes, kidneys, and adrenals may also be of value. Correlation of the history, clinical and necropsy findings, and, particularly, histopathologic changes should allow a definite diagnosis to be made, even in atypical cases.

SUMMARY

Grossly, the common signs of bovine malignant catarrhal fever (MCF) were poor condition, nasal and ocular discharge, corneal opacity, erosions of the muzzle, erythema of the udder and vulva, and, in a few cases, encrusting of the skin, tufting of the hair, and thickening and cracking of the epithelium of the teats. Several oral erosions were found in most affected animals. Diphtheric membranes were frequently observed in the nasal passages, pharynx, and trachea. Scattered areas of congestion, hemorrhage, and erosion were variably found in the digestive tract; they were usually most pronounced in the abomasum and large intestine when present. Lymph nodes, particularly those of the head, were enlarged, edematous, and hemorrhagic. Hemorrhage and erosion in the bladder were sometimes observed.

Microscopically, MCF was characterized by proliferative and infiltrative changes. In the case of squamous epithelial surfaces, these changes were followed by necrosis. The proliferative lesions were widely distributed, affecting vascular and epithelial structures. Marked elongation of the rete pegs was regularly observed in the squamous epithelium; adnexal skin structures often displayed marked hyperplasia. The dermal papillae contained abundant and pleomorphic cellular aggregations composed of monocytes, fibroblasts, lymphocytes, eosinophils, plasma cells, and mast cells. Similar proliferative and infiltrative processes were also seen in the laminae propriae of the conjunctivae, bronchioles, urinary bladder, gallbladder, ureters, and teat cistern; the epithelia of some of these structures appeared to be hyperplastic. Vascular proliferative and infiltrative lesions were present and marked in the liver and brain in each affected animal; they were less constantly found in most other organs. Proliferation in blood vessels was most commonly seen in the adventitia, but the media and endothelium were frequently involved. Mild to marked infiltrations of lymphocytes, monocytes, eosinophils, plasma cells, and mast cells were usually observed in and around the affected blood vessels.

Vesicles were frequently found in the squamous epithelium, often containing heterophils; they appeared to originate from coalescence of necrotic and ballooning cells in the stratum malpighii.

The lesions described are believed to be consistent and characteristic enough to be of diagnostic value. Intracytoplasmic inclusions in the neurons, previously reported by Stenius in Finland, were observed in the majority of affected animals; however, their specificity is questioned.

References

[1] Armed Forces Institute of Pathology: Manual of Histologic and Special Staining Technics. Washington, D.C., 1957.

[2] Berkman, R. N., and Barner, R. D.: Bovine Malignant Catarrhal Fever. I. Its Occurrence in Michigan. J.A.V.M.A., 132, (March, 1958): 243–248.

[3] Farquharson, J.: Malignant Catarrhal Fever. Vet. J., 102, (1946): 127–130.

[4] Goss, L. W., Cole, C. R., and Kissling, R. E.: The Pathology of Malignant Catarrhal Fever (Bovine Epitheliosis). Am. J. Path., 23, (1947): 837–841.

[5] Mallory, F. B.: Pathological Technique. 1st ed. W. B. Saunders Co., Philadelphia, Pa., 1938.

[6] Marshall, C. J., Munce, T. E., Barnes, M. F., and Boerner, F.: Malignant Catarrhal Fever. J.A.V.M.A., 56, (March, 1920): 570–580.

[7] North Dakota Livestock Sanitary Board: Malignant Catarrhal Fever. 31st Ann. Rep. North Dakota Livestock San. Board, (1937): 15–24.

[8] Rines, M. P., and Barner, R. D.: A Prolonged Case of Malignant Catarrhal Fever. M.S.C. Vet., 15, (1955): 108–109.

[9] Schofield, F. W.: The Value of a Histopathological Study of the Brain in an Atypical Case of Bovine Malignant Catarrh. Rep. Ontario Vet. College, Ontario Dept. Agric., (1950): 104–106.

[10] Smith, H. A., and Jones, T. C.: Veterinary Pathology. 1st ed. Lea & Febiger, Philadelphia, 1957.

[11] Stenius, P. I.: Bovine Malignant Catarrh. A Statistical Histopathological and Experimental Study. Inst. Path. Vet. College, Helsinki, 1952.

SUMMARIO IN INTERLINGUA

Maligne Febre Catarrhal Bovin in Michigan. II. Pathologia

Macroscopicamente, le lesiones commun de maligne febre catarrhal bovin (MFCB) esseva marcate perdita de peso, discarga nasal e ocular, opacitate corneal, erosiones del musello, erythema de ubere e vulva, e—in certe casos—incrustation del pelle, cespitositate del capillo, e spissification e fissuration del epithelio del mammillas. Sever erosion oral esseva constatate in le majoritate del afficite animales. Membranas diphtheritic esseva frequentemente observate in le passages nasal, le pharynge, e le trachea. Disperse areas de congestion, de hemorrhagia, e de erosion esseva trovate variabilemente in le vias digestive. Usualmente illos esseva pronunciate in le abomaso e le intestino crasse (in le casos in que illos esseva presente). Le nodos lymphatic, specialmente in le capite, esseva allargate, edematose, e hemorrhagic. Hemorrhagia e erosion in le vesica esseva observate in plure casos.

Microscopicamente, MFCB esseva characterisate per alterationes proliferative e infiltrative. In le caso de superficies epithelial squamose, iste alterationes esseva sequite per necrosis. Le lesiones proliferative esseva distribuite extensemente e afficeva structuras vascular e epithelial. In le epithelio squamose, accumulationes cellular in le papillas esseva observate regularmente. Le adnexe structuras cutanee revelava marcate grados de hyperplasia in multe casos. Le aggregationes cellular in le papillas dermal esseva abundante e de character pleomorphic, incluse monocytos, fibroblastos, lymphocytos, eosinophilos, cellulas plasmatic, e mastocytos. Simile processos proliferative e infiltrative esseva etiam vidite in le laminas proprie del oculo, del bronchiolos, del vesica urinari, del vesica biliari, del ureteres, e del cisterna mammillari. Le epithelios de certes de iste structuras pareva esser hyperplastic. Lesiones vascular de character proliferative e infiltrative esseva presente e marcate in le hepate e le cerebro de omne le afficite animales. In altere organos, illos esseva trovate minus uniformemente. In le vasos de sanguine, proliferation esseva incontrate le plus communmente in le tunica adventitie, sed le media e le endothelio esseva etiam afficite in frequente casos. Leve o marcate grados de infiltration per lymphocytos, monocytos, eosinophilos, cellulas plasmatic, e mastocytos esseva usualmente observate in e circum le afficite vasos de sanguine.

Vesiculos esseva frequentemente incontrate in le epithelio squamose. In multe casos illos contineva heterophilos. Illos pareva haber lor origine in le coalescentia de cellulas necrotic con cellulas ballonante in le strato de Malpighi.

Le hic describite lesiones pare esser sufficientemente uniforme e characteristic pro esser de valor diagnostic. Inclusiones intracytoplasmic in le neurones, previemente reportate per Steinus in Finlandia, esseva notate in le majoritate del animales afficite, sed le specificitate de iste phenomeno es questionate.

Correction—Infectious Bovine Keratoconjunctivitis

In the article, "Infectious Bovine Keratoconjunctivitis. I. Etiology," by J. B. Henson and L. C. Grumbles, which appeared in the September, 1960, issue of the *American Journal of Veterinary Research*, pp. 761–766, the second sentence under Results (page 764) should read "*A total of 86 isolations were made from 93 animals in 15 herds.*"

Studies of Bovine Portal Blood. I. Establishment and Maintenance of Portal and Mesenteric Vein Catheters

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THE PROCUREMENT of blood in large volumes and at frequent intervals from the portal vein of conscious calves opens many avenues for research in animal nutrition and physiology. Methods for obtaining blood from the portal circulation of cats,[2] dogs,[7,8] and sheep[1,9,11] have been described. Using copper-constantan thermocouples, Fegler and Hill[4] estimated the portal-vein blood flow in anesthetized sheep to be approximately one liter per minute. Conrad *et al.*[3] were successful in obtaining serial blood samples from the portal system (gastrosplenic vein) of calves.

The purpose of this report is to describe our technique for obtaining portal-vein blood from unanesthetized calves. The method described was used successfully for collecting portal blood samples up to 73 days postoperatively. The surgical procedure is patterned in part after the method used by Jungblut and co-workers[7] on dogs.

Within the scope of this experiment, 15 dairy calves, weighing 112 to 240 kg., were used. On these animals, a total of 25 operations were performed to insert cannulas into the portal vein or the mesenteric vein (used for injecting dye in flow determinations, Fries and Conner[5]). Not all of the operations turned out favorably. Some postoperative difficulties encountered were kinked tubes, coagulation of blood within a vein or cannula, pylethrombophlebitis (fig. 1), and over-dosing with an anticoagulant. The physiologic data obtained from studies on the portal vein blood will be presented in other articles.[5,6]

From the Department of Surgery and Medicine (Conner) and the Department of Dairy (Fries), Michigan State University, East Lansing. Published with the approval of the director of the Michigan Agricultural Experiment Station as Journal Article No. 2519. Dr. Fries is now with the Dairy Cattle Research Branch, USDA, Montana State College, Bozeman.

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EXPERIMENTAL PROCEDURE

PREANESTHETIC MEDICATION AND ANESTHESIA

Access to the portal vein was gained via entry into one of the veins in the mesentery of the small intestine, either ileum or jejunum. Most operations were performed with the calves in lateral recumbency (left side down) and depressed by 2 per cent thiamylal sodium* given intravenously. A preoperative injection of one of the ataractic drugs seemed to lessen the amount of thiamylal sodium needed and also served to keep the animal quiet during the first ten to 12 hours following the operation.

Several operations were started under paravertebral lumbar nerve block anesthesia with the animal standing. However, when the vein was opened, and especially while the cannula was being passed through it, the animals expressed severe pain by jumping, kicking, and even lying down. These conditions made it nearly impossible to perform the delicate task of entering one of the small mesenteric veins. A local anesthetic, 1 per cent lidocaine** or 2 per cent procaine hydrochloride, injected on both sides of the vein for a distance of several centimeters, did not alleviate the pain, although it was lessened. Probably in adult cattle the standing operation would be preferred. With more proficient administration of a local anesthetic in the mesentery, perhaps all evidence of pain could be eliminated.

SURGICAL PROCEDURE

The peritoneal cavity was entered through a vertical abdominal incision located low in the right paralumbar fossa. The incision started approximately at the apex of the triangle which outlines the paralumbar fossa and extended 8 to 10

* Surital Sodium, produced by Parke-Davis Co., Detroit, Mich.

** Xylocaine, produced by Astra Pharmical Co., Worcester, Mass.

inches downward. A loop of ileum or jejunum was brought to the outside, and several inches of a vein in the mesentery was exposed by carefully incising the mesentery over it and by gently dissecting away most of the perivenous fascia.

Initially, entry into the vein was attempted through the lumen of a hypodermic needle which had been inserted into the vein. Because of the size of needle needed and the extreme fragility of the vein, this procedure proved to be unsatisfactory and was soon abandoned. The preferred technique consisted of making a transverse incision halfway across the vein by inserting a small, curved, cutting, suture needle transversely through the vessel and by cutting down on the needle with a scalpel if necessary. Into this rent in the vein a small, blunt-pointed probe was introduced, stabilizing the vein and serving as a guide for the introduction of the polyethylene catheter.

Retaining a loop of intestine outside the peritoneal cavity may cause an acute angulation of the veins leading into the larger radicles of the portal system. For ease of passing the catheter through the vein, it was sometimes necessary to change the position of the exposed intestine or perhaps even to partially replace it into the cavity. Location of the end of the catheter was determined by palpation of the portal fissure (porta hepatis). The desired location for the tip of the portal vein catheter was approximately 1 inch before the major branching of the vein within the liver. This site is beyond the origin of the gastrosplenic vein. After the catheter was in the desired location, several ligatures of nonabsorbable material were snugly tied around the vein and catheter near its point of entry. Further anchoring of the catheter was accomplished by suturing it to the mesentery.

Fig. 1—Pylethrombophlebitis in a portion of liver at the porta hepatis. The opened portal vein (A) has a thrombus (B) attached to its lining. The thrombus was near the end of an indwelling polyethylene catheter in the portal vein.

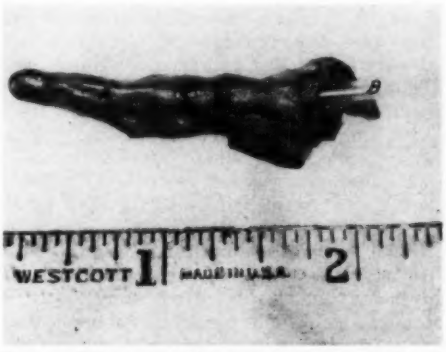

Fig. 2—Fibrin clot (A) that formed around and over the end of an indwelling mesenteric vein catheter (B).

In all portal vein catheterizations, a second catheter was also introduced into a branch of the mesenteric vein fairly close to the insertion site of the portal vein catheter. The mesenteric catheter was used for injecting dyes to determine volume flow through the portal vein. The end of the mesenteric catheter, therefore, must be posterior to the site of convergence of the gastrosplenic vein and the portal vein.[9]

In the first experimental animals, only a short length (2 to 3 inches) of catheter was introduced. Invariably, these catheters did not operate after two to three days. Some became completely impassable to fluid, or fluid could be injected through it but not all reached the portal blood and what did was slow in arriving. Upon necropsy, there was intravascular clotting about the indwelling catheter and occlusion of the vein for some distance beyond the end of the catheter (fig. 2. A logical explanation for this was that ligation of the vein containing the catheter prevented blood flow through it. If the end of the catheter was in this stagnant blood, the inevitable clotting would, of course, involve

it. Inserting the catheter beyond a point where one or more tributaries enter the vein minimized the problem of having intravascular clotting involve the end of the catheter.

After completing the catheterizing process, the intestines were returned to their normal position and the two catheters were brought through the abdominal wall via separate stab incisions in approximately the center of the right paralumbar fossa. To avoid kinking of the catheters in the peritoneal cavity, very little extra catheter was left in the cavity. In one of the early operations, when 5 or 6 inches of slack catheter was left in the cavity, blockage was encountered due to a kink in the catheter. Catheters were fixed externally by a ligature anchored in the skin. Each catheter was equipped with a blunt-pointed hypodermic needle fitted snugly in its lumen. A 3-inch piece of rubber tubing was attached to the needle by means of a luerlock fitting. The free end of the rubber tubing also carried a fitting to which a syringe could be attached (fig. 3). The

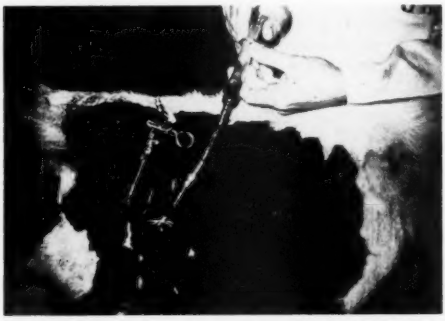

Fig. 3—Right side of a 240-kg. Holstein-Friesian calf showing the mesenteric and portal vein catheters after their emergence from the abdomen in the paralumbar fossa. The catheter with syringe attached is in the portal vein. The mesenteric vein catheter illustrates the method of clamping.

external portion of the catheters with their extensions of rubber tubing were secured to the animal with adhesive tape in a manner to avoid acute angulation of the catheter.

CARE OF CATHETERS

All catheters consisted of polyethylene tubing which was siliconized inside and outside prior to use. The polyethylene tubing varied in size from those having an inner diameter of 0.034 inch and an outer diameter of 0.050 inch to those having an inner diameter of 0.070 inch and an outer diameter of 0.110 inch. The portal vein catheter had to be large enough to permit withdrawal of 2 ml. of blood at two-second intervals. For this, a catheter with an inner diameter of 0.062 and an outer diameter of 0.082 inch usually was used. The smaller caliber tubing served well for injection purposes (mesenteric catheter). During the process of inserting catheters through the veins, it was necessary to keep them completely filled with an anticoagulant solution. On several occasions, heparinized saline solution was slowly dripped through the catheters during their insertion.

When not in use, it was essential to have the catheter filled with an anticoagulant solution. A 2.5 per cent solution of sodium citrate or a heparinized saline solution were equally satisfactory. The short piece of rubber tubing attached to the catheter simplified the injection of anticoagulant solution into the catheter. While it was being injected, a pinch-clamp was placed on the rubber tubing and left there. This technique eliminated the possibility of blood flowing back into the catheter, which could happen with other methods of closing the external end of the catheter.

The catheters were checked for patency every day. After each withdrawal of blood from the catheters, they were flushed routinely with physiologic saline solution before instilling the anticoagulant solution.

It is felt that much of our success in keeping the catheters open for long periods of time centered around three procedures: (1) daily checking of the catheters by withdrawing blood, (2) preventing the backflow of blood into the catheters, and (3) administration of anticoagulant drugs to the calves. The latter probably had more significance during the first postoperative week than it did later. Nevertheless, on 2 animals, the anticoagulant drug was given throughout the duration of the experiment (6 and 10 weeks).

ANTICOAGULANT DRUGS

Repository heparin sodium,* a long-acting anticoagulant, was used alone in 1 calf and in conjunction with another antico-

* Depo-Heparin Sodium, Upjohn Co., Kalamazoo, Mich.

agulant in 2 other calves. Since this drug has a prolonged absorption time, there is a latent period between the time of injection and the time of desired anticoagulant activity. A blood clotting time of 34 to 40 minutes was judged desirable. For a 114-kg. calf, daily injections of 400 mg. of repository heparin sodium were necessary to maintain this level of clotting activity.

Bishydroxycoumarin,** a prothrombinopenic drug, was used as an anticoagulant on 5 calves. After the initial dose, there was approximately a 48-hour latent period before the occurrence of the desired anticoagulant activity, as judged by prothrombin clotting time with whole plasma. Prothrombin times were determined daily and served as a guide for ascertaining the dosage. Untreated calves gave prothrombin times from 16 to 20 seconds with the thromboplastin used. The dosage of bishydroxycoumarin for treated calves was regulated to keep the prothrombin time in the range of 40 to 50 seconds. Two calves hemorrhaged internally when the prothrombin time was in excess of 70 seconds for several days. Vitamin K† injections and blood transfusions saved the life of 1 calf, but the other died.

Administration of bishydroxycoumarol was begun 40 hours before the operation because of its latent period. The immediate postoperative regimen usually included the intravenous infusion of 100 to 200 mg. of aqueous heparin. If subsequent operations on treated calves were necessary, the administration of bishydroxycoumarol was stopped and the operation was attempted only after the prothrombin time had fallen below 25 seconds.

A Possible Sequela

During blood flow determination studies on 1 calf (13c), evidence was gathered to indicate that the portal vein catheter which was originally placed in the porta hepatis had changed position and was located posterior to the site of entrance of the gastrosplenic vein. In this animal, the dye-injection catheter was located in a branch of the ruminal vein. Dye injected into this calf was present in blood from the carotid artery (exteriorized carotid loop) before being present in blood taken from the portal catheter. At necropsy, the portal catheter was posterior to the point of entrance of the gastrosplenic vein. However, the catheter was not posterior to any of the other major tributaries to the portal system. In none of the other 14 animals was there evidence of shifting of position of the catheters after their initial placement.

** Dicumarol is the registered collective trade mark adopted by the Wisconsin Alumni Research Foundation for bishydroxycoumarin.

† Vitamin K Analogue, Upjohn Co., Kalamazoo, Mich.

Summary

Portal blood samples were collected successfully for periods of up to 73 days in 15 unanesthetized calves in which portal-vein catheters had been established. Using a special technique, both portal-vein and mesenteric-vein catheters were established in calves. There were no untoward effects due to the catheters and the calves were apparently normal during the period of experimentation.

Evidence indicated that in 1 of the 15 calves, the portal-vein catheter changed position following its initial placement in the porta hepatis.

References

[1] Annison, E. F., Hill, K. J., and Lewis, D.: Portal Blood Analysis in the Study of the Absorption of the Ruminal Fermentation Products. Biochem. J., 60, (1955): 19.

[2] Bessman, S. P., Magnes, J., Schwerin, P., and Waelsch, H.: The Absorption of Glutanic Acid and Glutamine. J. Biol. Chem., 175, (1948): 817.

[3] Conrad, H. R., Smith, H. R., Vandersall, J. H., Pounden, W. D., and Hibbs, J. W.: Estimating Gastrosplenic Blood Flow and Volatile Fatty Acid Absorption from the Forestomachs of Calves. J. Dai. Sci., 41, (1958): 1094.

[4] Fegler, G., and Hill, K. G.: Measurement of Blood Flow and Heat Production in the Splanchnic Region of Anesthetized Sheep. Quart. J. Exptl. Physiol., 43, (1958): 189.

[5] Fries, G. F., and Conner, G. H.: Studies of Bovine Portal Blood. II. Blood Flow Determinations and Hemodilution Factors in the Portal Vein. Am. J. Vet. Res., In press.

[6] Fries, G. F., and Conner, G. H.: Observations on Absorption of Glucose into the Portal Vein of Calves. J. Dai. Sci.: In press.

[7] Jungblut, P. W., Lohmann, B., Schober, R., and Turba, F.: Eine Methode zur fortlaufenden Blutentnahme aus der Vena Portae von Hunden. Experientia, 11, (1955): 241.

[8] London, E. S.: Angiostomie und Organestoffwechsel. Des All-Union Instituts für exper. Med., Moscow, 1935.

[9] Schambeye, P.: Volatile Acids and Glucose in Portal Blood of Sheep. 2. Sheep Fed Hay and Hay Plus Crushed Oats. Nord. Vet.-med., 3, (1951): 748.

[10] Schambeye, P.: Experimental Estimation of the Portal Blood Flow in Sheep. I. Examination of an Infusion Method and Results from Acute Experiments. Nord. Vet.-med., 7, (1955): 961.

[11] Schambeye, P., and Phillipson, A. T.: Volatile Fatty Acids in Portal Blood of Sheep. Nature, 164, (1949): 1094.

SUMMARIO IN INTERLINGUA

Studios de Sanguine Portal Bovin. I. Establimento e Mantenentia de Catheteres de Vena Portal e de Vena Mesenteric

Specimens de sanguine portal esseva colligite a bon successo durante periodos de usque a 73 dies in 15 non anesthesiate vitellos in que catheteres de vena portal habeva essite establite. Per medio de un technica special, catheteres tanto de vena portal como etiam de vena mesenterie esseva establite in vitellos. Nulle adverse effectos esseva causate per le catheteres, e le vitellos esseva apparentemente normal durante le periodo del experimentation.

In 1 del 15 vitellos, le evidentia indicava que le catheter porto-venal cambiava su position post su insertion initial in le porta de hepate.

Studies of Glycine-1-C^{14} Metabolism in the Dairy Cow—Urinary Hippuric Acid and Pulmonary CO_2 Excretion

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PREVIOUS STUDIES from this laboratory have reported the use of glycine-1-C^{14} in the measurement of serum protein turnover rates in dairy cows.[7] These investigations have demonstrated that an average of 3.7 per cent of the injected C^{14} was initially incorporated into the extracellular serum protein pool, with serine and glycine accounting for 80 to 90 percent of the total activity.

Berlin *et al.*[4] studied the rate of decrease of specific activity of the urinary hippuric acid in man after the administration of glycine-2-C^{14} and sodium benzoate. This decrease was described in terms of two exponential components having half-times of 6.8 and 75.0 days. The rates for these two components agreed closely with the rates of the two slowest components of the pulmonary $C^{14}O_2$ excretion. The $C^{14}O_2$ excretion was quite rapid, with 40 per cent eliminated in one subject in 24 hours and nearly 60 per cent by the fifth day.[5] By 55 days after injection, 83 to 92 per cent of the injected C^{14} was excreted via the lungs in four subjects. Henriques *et al.*[8] measured the free glycine-2-C^{14} in plasma, liver, and muscle from five minutes to 18 hours after its intravenous injection into rabbits and suggested that the rate of decrease of specific activity of the plasma glycine almost certainly occurred by three or more reactions proceeding with greatly different rates. By applying the method of least squares to portions of the descending curve of the specific activity of the plasma glycine, an equation of the "three-compartment" type of Solomon [15] was formulated.

This paper presents data from investigations with 3 dairy cows given glycine-1-C^{14} intravenously. The cumulative excretions of C^{14} as pulmonary $C^{14}O_2$ and glycine-C^{14} from the urinary hippuric acid are presented. The decrease in their specific activities was estimated in terms of several rate processes.

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This investigation presents results obtained by the Davis Tracer Team for Metabolic Research, supported in part by grants from the United States Atomic Energy Commission and the National Science Foundation.

MATERIALS AND METHODS

Glycine-1-C^{14} was injected intravenously and urine samples were collected for hippuric acid isolation as described previously.[7] Total urine was collected for the first 48 hours, followed by random sampling every few days until the trial was complete. Data pertaining to these animals and the amount of isotope administered is shown (table 1). The respiratory apparatus and the collection of the respired CO_2 have been previously described by Kleiber and Edick,[10] as have the preparation and combustion of samples and the counting for their radioactivity.[11]

TABLE 1—Data from Experimental Trials with Cows Given Glycine-1-C^{14}

Cow No.	Age (years)	Weight (kg.)	Injected dose (mc.)	Relative dose (μc./kg.)	State
57	5.0	541	3.75	6.94	Lactating
78	4.5	449	4.26	9.48	Lactating
917	Mature	383	1.50	3.92	Nonlactating

A "transfer quotient" was calculated in estimating the quantity of respiratory CO_2-carbon that may have originated from the carbon-1 atom of glycine. The transfer quotient has been formulated by Kleiber[9] as the ratio of the carbon from a given precursor to the total carbon in a product. This calculation is independent of the many intermediary processes that may be involved in the transfer, providing the precursor metabolite is the only original source of C^{14}. If no delay is involved in the metabolic transfer, the transfer quotient (q) can be expressed as[9]:

$$q = \frac{Sp}{S} = \frac{\int_o^t \sigma dt}{\int_o^t \pi dt}, \text{ where } S = \text{rate of total C}$$

transfer to the product, Sp = rate of C transfer to the product from a given precursor, σ = specific C^{14} activity in the product, and π = specific C^{14} activity in the precursor.

RESULTS AND DISCUSSION

Pulmonary $C^{14}O_2$ Excretion.—The standard specific activities of $C^{14}O_2$ following injection of the glycine-1-C^{14} are presented (fig. 1). Maximum specific activity of the $C^{14}O_2$ for both lactating cows occurred at 30 minutes after injection, as compared to 22 minutes for the nonlactating cow. The change in the specific activity of the respired CO_2 during the phase of descending radioactivity can be described in terms of a series of rate processes, each of a "first order" type, as previously described by Berlin *et al.*[5] for man using glycine-2-C^{14}. In the 2 lactating cows, the decrease in specific $C^{14}O_2$ activity was expressed as two components during the first 24 hours after injection. Regression coefficients were calculated by the method of least squares from the logarithms of the standard specific activities (λ).* After extrapolation of the slower component to injection time (t = hours), differences of the antilogarithms between the extrapolated and original curve were calculated for each sample period. A new series of the logarithms of the differences in activities was plotted again against time, and the procedure was repeated until both first order components had been calculated. The equations calculated from the experimental data were:

$\lambda = 9.10e^{-0.614t} + 0.496e^{-0.0211t}$ (Cow 57)

$\lambda = 7.52e^{-0.685t} + 0.983e^{-0.0744t}$ (Cow 78)

Half-time ($T\frac{1}{2}$) values for these components were 1.13 and 14.24 hours in lactating cow 57 and 1.01 and 9.30 hours in lactating cow 78. In nonlactating cow 917, standardized specific activity (λ) of the respired CO_2 was also determined* at 73.0 hours (0.136), 174.0 hours (0.042), and at 29 days 3.0 hours (8.38×10^{-3}).

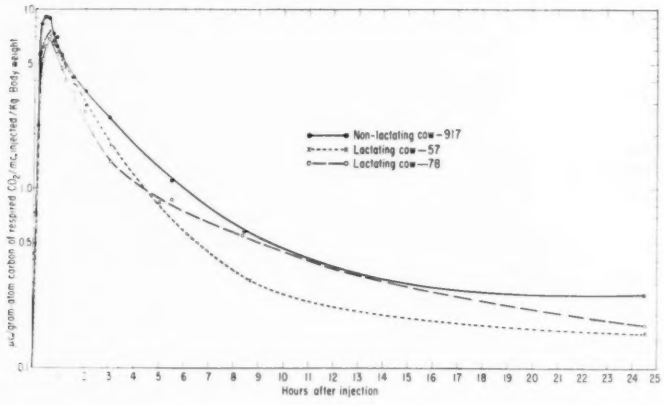

Fig. 1—Standard specific activities of respired CO_2 form glycine-I-C^{14}, showing increasing and decreasing components in the first 24 hours.

From this data, it was possible to calculate two slower components in addition to the rapid components. The exponential equation for the decrease in the standardized specific activity of the respiratory $C^{14}O_2$ in the nonlactating cow was:

$$\lambda = 8.65e^{-1.72t} + 5.89e^{-0.370t} + 0.301e^{-0.0187t} + 0.071e^{-0.0031t}$$

These four components had half-times ($T\frac{1}{2}$) of 0.4 hour, 1.87 hours, 36.9 hours, and 9.43 days. Since the respiratory $C^{14}O_2$ of the lactating cows was not assayed at these later periods, no slower components could be calculated. The inclusion of the slower components tends to shorten the calculated $T\frac{1}{2}$ values of the more rapid components.

Cumulative pulmonary $C^{14}O_2$ excretion is presented (fig. 2). By three hours after injection, the nonlactating cow had expired 11.5 per cent of the injected dose as compared with 10.8 and 12.2 per cent for lactating cows 78 and 57, respectively. At 32 hours, both lactating cows had expired 20.1 per cent of the injected dose as $C^{14}O_2$,

* Standard specific activity = $\frac{\mu c./gaC}{\mu c. \text{ injected/kg. body wt.}}$

* The authors are indebted to B. M. Tolbert and the Donner Laboratory of Medical Physics, University of California, Berkeley, for analysis of these samples in an ionization chamber calibrated with the current Oak Ridge C^{14} millicurie by specially prepared samples.

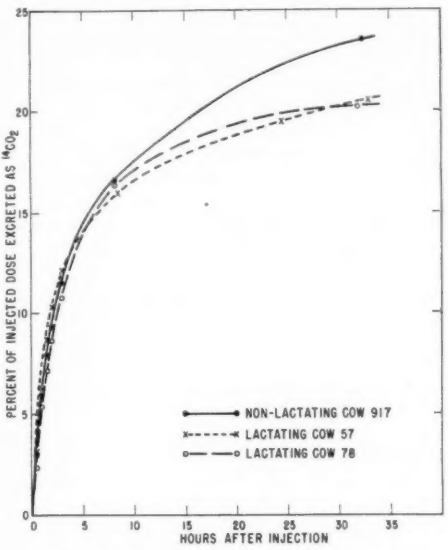

Fig. 2—Cumulative C^{14} excretion as $C^{14}O_2$.

while the nonlactating cow had expired 23.5 per cent. Cumulative excretion of $C^{14}O_2$ in man, as reported by Berlin *et al.*[5] was quite rapid following glycine-2-C^{14}; 40 per cent was eliminated in one subject in 24 hours, followed by a total of 60 per cent by the fifth day. In comparison, lactating cows excreted 19.5 per cent of glycine-1-C^{14} in 24 hours, while the nonlactating cow had excreted 21 per cent. Nonlactating cow 917 excreted 42.3 per cent of the administered dose thirty days after injection.

Hippurate glycine-C^{14} Excretion.—The standard specific activities of the urinary hippurate-glycine for lactating cows 57 and 78 and nonlactating cow 917 are shown (fig. 3). The nonlactating cow appeared clinically febrile (104.0 C.) six days after the injection of the labeled glycine. Data prior to the sixth day are considered representative of the normal state, with samples after six days becoming progressively more affected by the pathologic changes until death at 54 days. Since anorexia was present after ten days, a negative nitrogen balance most likely was present.

The decrease in radioactivity of the urinary hippuric acid-glycine with time, which was rapid at first and considerably slower later, indicates that the glycine is mixed and metabolized by several reactions of different rates. Barton[2] injected glycine-2-C^{14} into mice and measured the C^{14}-formaldehyde liberated by ninhydrin as an index of the specific activity of glycine, assuming formaldehyde came exclusively from glycine. He observed that, in 15 to 20 minutes, the radioactivity values of the

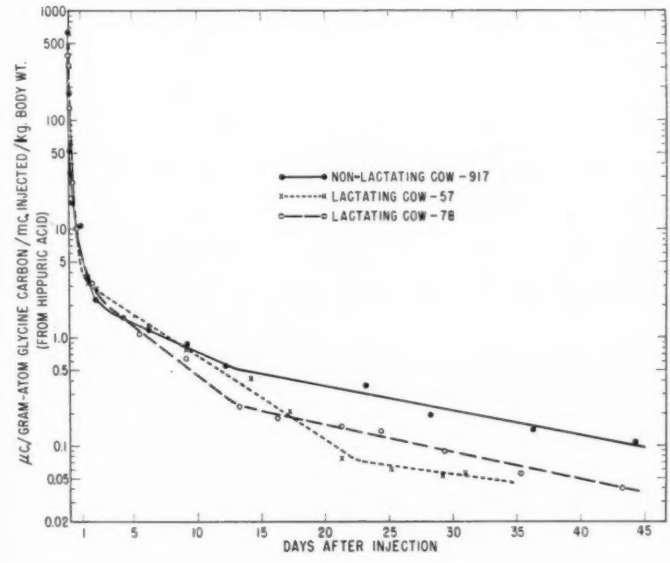
μC/GRAM-ATOM GLYCINE CARBON/mC. INJECTED/kg. BODY WT.
(FROM HIPPURIC ACID)
DAYS AFTER INJECTION
NON-LACTATING COW - 917
LACTATING COW - 57
LACTATING COW - 78

Fig. 3—Standard specific activities of glycine carbon from urinary hippuric acid.

free glycine of liver, muscle, and blood were equal. Contrary to this report, Arnstein and Neuberger [1] found equilibration of free glycine between the liver and other tissues of the rat to be a relatively slower process than previously reported. Henriques *et al.*[8] isolated the free glycine-1-C^{14} in rabbits as 2:4-dinitrophenylglycine using buffered celite chromatography. They inferred that complete replacement of the free liver glycine pool by free plasma glycine occurred between 15 and 20 minutes. They concluded that permeability barriers play only an insignificant part in this exchange, with the rate of blood flow being the limiting factor.

Simkin and White [13] have shown in the rat and rabbit that increasing the oral dose of benzoate, which is excreted as hippuric acid, leads to an exponential decrease of the concentration of free glycine in the liver and plasma. No changes in the concentration of liver protein glycine were observed. In addition, they found only insignificant changes in the concentration of peptide glycine and concluded that most of the glycine used in conjugation with hippuric acid was provided via the free glycine of the liver. This free liver glycine was replenished from sources such as its liver synthesis from precursors and by the liberation of bound glycine.

Snapper *et al.*[14] found only limited hippuric acid synthesis in perfused kidneys of ruminants, as compared with greater synthesis in the kidneys of human beings, pigs, and dogs. The studies of Bunge and Schmiedeberg,[6] followed by corroborating studies of Koch [12] and Bashford and Cramer,[3] have been interpreted as evidence that the kidneys of herbivores are of limited importance, as compared to the liver, in hippuric acid synthesis.

Since exchanges between plasma and liver may be a rapid process, the glycine isolated as hippuric acid a very short time following intravenous injection may nearly equal the free plasma glycine in specific activity. This situation assumes no great delay in the conjugation and excretion of hippuric acid. After the intravenous injection of 30 Gm. of buffered sodium benzoate into non-lactating cow 917 prior to the trials under study, concentrations of eight times the pre-injection urinary levels of hippuric acid appeared within 30 minutes. Intravenous injections of up to 6 Gm. of glycine (at 100 mg. per minute) had no measurable effect on the urinary hippurate excretion rate. In nonlactating cow 917 and lactating cow 57, 33 and 51 per cent, respectively, of the total 30-day cumulative excretion of the radioactivity of the urinary hippurate-glycine was present within the first hour, indicating a very rapid plasma-liver glycine exchange and hippuric acid excretion.

The values for the specific activities of the urinary hippurate-glycine in this study of dairy cows cannot be expressed as a single exponential equation. Henriques *et al.*,[8] making a comparable study in rabbits, applied the method of least squares to the curve of the specific activity of the hippurate-glycine during the first 18 hours after injection. They suggested their curve fit the three-compartment curve of Solomon [15] fairly well. Although no quantitative or physiologic significance could be attached to their calculations, they estimated that the decrease of radioactivity in the plasma glycine occurred almost certainly by three or more reactions proceeding at greatly different rates. Since the glycine of the hippuric acid was assayed for radioactivity in cows 917, 56, and 78 for 44.3, 31.0 and 43.3 days, respectively, we calculated from our trials a fourth, slower component in addition to the three more rapid components.

In the calculation of the four components, the slowest was first computed as a least squares regression line, selecting the last four or five specific activity values that

TABLE 2—Half-Time Values for Components Representing the Disappearance of Hippurate-Glycine Specific Activities and the Magnitude of Glycine Pools Calculated by the Isotopic Dilution Technique

Cow No.	Stage of lactation	Component $T_{1/2}$ and pool size per kilogram of body weight							
		1		2		3		4	
		Hours	mg.	Hours	Gm.	Days	Gm.	Days	Gm.
917	Nonlactating	0.52	38.5	8.22	1.09	2.99	15.0	15.60	48.9
57	Lactating	0.73	51.0	4.41	0.615	3.64	8.95	29.47	327.5
78	Lactating	0.85	57.0	5.63	0.679	2.25	8.16	11.48	71.6

followed a "first order" process when plotted on semilogarithmic coordinates. After the extrapolation of this slowest component to injection time, differences of the antilogarithms between the extrapolated and original curve were calculated for each sample period. A new series of the logarithms of the differences in activities was plotted again against time and the procedure repeated until a series of "first order" components had been calculated.

The equations were calculated from the experimental data derived from the standard specific activities of the descending curve of the radioactivity of the hippuric acid-glycine (fig. 3).

Nonlactating cow 917

$$\lambda = 986e^{-1.24t} + 34.4e^{-0.0942t} + 2.52e^{-9.66 \times 10^{-2}t} + 0.766e^{-8.29 \times 10^{-4}t}$$

Lactating cow 57

$$\lambda = 737e^{-0.945t} + 81.4e^{-0.357t} + 4.16e^{-7.92 \times 10^{-2}t} + 0.114e^{-9.78 \times 10^{-4}t}$$

Lactating cow 78

$$\lambda = 658e^{-0.851t} + 55.3e^{-0.129t} + 4.60e^{-1.28 \times 10^{-2}t} + 0.524e^{-2.51 \times 10^{-3}t}$$

Since urine was withdrawn at only hourly intervals early in the trials when the greatest change of specific activity was occurring, there was a limit to the number of components that could be calculated. The first component, however, would be an average of all the rapid components present in the early mixing process. This first rapid mixing component was probably from a rapid exchange in vascular, interstitial, and some cellular compartments soon after injection. The second component of diminishing glycine specific activity could well be influenced by the metabolism of proteins and peptides with fast turnover rates or by processes such as hippuric acid formation and lactation. After a few days, the descending curve could only be interpreted as the sum total of all the processes utilizing glycine in either synthetic or catabolic reactions or excreting glycine from the free "glycine pool" which is in continuous exchange with glycine utilized in benzoic acid conjugation.

Great care must be taken in the physiologic interpretation of such curves constructed as the sum of exponents. With limited data, certain arbitrary decisions necessary for the calculation of components and their subsequent subtraction from other values could affect the final exponential equation considerably. Assumptions about the number of compartments existing due to the results of such exponential curves is conjectural, since different compartments could have similar rates of mixing or utilization.

Half-time values for the four components are given (table 2). From these half-time values, it is apparent that the second component accounts mainly for the differences between lactating and nonlactating cows in the loss of specific activity from the free glycine pool. The shorter half-time of the second component in the lactating cows could result from the rapid utilization of glycine and other compounds in exchange with glycine for milk synthesis.

By extrapolating the components to injection time, a pool size corresponding to this turnover process can be estimated by isotope dilution (table 2). Nonlactating cow 917 had a glycine pool corresponding to the first rapid components of 38.5 mg. per kilogram as compared with 51.0 and 57.0 mg. per kilogram in lactating cows 57 and 78, respectively. The existence of these greater glycine pools in cows 57 and 78 in the early mixing process could be attributable to the physiologic state of lactation, since both increased blood volume and cellular mammary tissue are obvious additions. Data presented suggest that pool 2 in the lactating cows has both a greater turnover rate and a smaller size than that of the nonlactating cow. The size of pool 3 again was of less magnitude in cows 57 and 78 as compared with cow 917; however, $T\frac{1}{2}$ values of the glycine were similar. Data pertaining to pool 4 are difficult to interpret, since nearly as much variation exists between the lactating cows as exists between the lactating and nonlactating cows. The febrile condition of nonlactating cow 917 could have affected the turnover rate of the free glycine pool six days postinjection. Any errors in the extremely slow turnover process, however, would have insignificant effects on the other components.

Glycine pool 4, as measured in cow 78, was 7.16 per cent of the body weight. Since glycine obviously does not amount to 7.16 percent of the body weight, the concept of a glycine pool merits further consideration. Each glycine pool measured by this method would include such metabolites as are in continuous exchange with the free liver glycine. It is quite apparent that the gly-

cine pool includes more than free glycine. Glycine pools with slower turnover rates could include exchanging metabolites with correspondingly slower turnover rates. The glycine pools corresponding to each turnover process could include different metabolites as well as similar metabolites from different body sources.

Calculations concerning the cumulative excretion of hippuric acid-glycine indicated that lactating cow 57 excreted 11.3 per cent of the injected dose as hippuric acid-glycine in 31 days; lactating cow 78 excreted 10.0 per cent in 43.2 days, and the nonlactating cow excreted 8.02 per cent in 44.2 days. These values were calculated by using an average daily hippurate excretion, determined over a 45-hour period with cows on a constant diet of alfalfa hay. Feed consumption and composition was maintained constant during all trials, except in nonlactating cow 917 six days after injection.

A transfer quotient was calculated to estimate the quantity of respiratory CO_2-carbon that could have originated from the carbon-1 atom of the free glycine pool. In this estimation, it was assumed that the glycine isolated from the urinary hippuric acid was representative of the specific activity of the free plasma glycine pool during the first 33 hours after injection. It was also assumed that no appreciable delay in the metabolic transfer occurred. The average per cent ($q \times 100$) of the respiratory CO_2 that may have originated from the carbon-1 atom of glycine was 1.09 per cent for the nonlactating cow and 0.78 and 0.85 per cent for lactating cows 57 and 78, respectively.

SUMMARY

1) Three dairy cows were given intravenous injections of glycine-1-C,[14] and the radioactivity in the pulmonary Co_2 and the urinary hippuric acid-glycine was measured.

2) The time curves of the standard specific activities of the CO_2 and hippurate glycine are expressed both as cumulative excretion and, mathematically, as the sum of several "first order" components.

3) Two lactating cows and 1 nonlactating cow had expired 20.1, 20.1, and 23.5 per cent respectively of the injected dose of glycine-1-C [14] as respiratory CO_2, by thirty-two hours after injection.

4) Approximately 7 and 9 per cent respectively, of the injected dose of the glycine-C [14] was excreted as urinary hippuric acid-glycine by 2 lactating dairy cows within 24 hours after injection. A nonlactating dairy cow excreted approximately 5 per cent of the injected glycine as hippuric acid by 24 hours post injection.

References

[1] Arnstein, H. R. V., and Neuberger, A.: Hippuric Acid Synthesis in the Rat. Biochem. J., 50, (1951): 154.

[2] Barton, A. D.: Metabolism of Glycine. Proc. Soc. Exptl. Biol. & Med., 77, (1951): 481.

[3] Bashford, E., and Cramer, W.: Über die Synthese der Hippursäure im Thierkörper. Ztschr. physiol. Chem., 35, (1902): 324.

[4] Berlin, N. I., Hewitt, C., and Lotz, C.: Hippuric Acid Synthesis in Man After the Administration of -C^{14} Glycine. Biochem. J., 58, (1954): 498.

[5] Berlin, N. I., Tolbert, B. M., and Lawrence, J. H.: Studies in Glycine-2-C^{14} Metabolism in Man. I. The Pulmonary Excretion of $C^{14}O_2$. J. Clin. Invest., 30, (1951): 73.

[6] Bunge, G., and Schmiedeberge, O.: Über die Bildung der Hippursäure. Arch. exper. Path. u. Pharmakol., 6, (1876): 233.

[7] Cornelius, C. E., Black, A. L., and Kleiber, M.: The Use of Glycine-1-C^{14} in the Measurement of Serum Protein Turnover Rates in Dairy Cows. Am. J. Vet. Res., 20, (Jan., 1959): 44.

[8] Henriques, O. B., Henriques, S. B., and Neuberger, A.: Quantitative Aspects of Glycine Metabolism in the Rabbit. Biochem. J., 60, (1955): 409.

[9] Kleiber, M.: Precursor—Product Relationship for Milk Formation in the Intact Dairy Cow. Extrait de la Rev. canad. Biol., 13, (1954): 333.

[10] Kleiber, M., and Edick, M.: A Respiration Apparatus for C^{14} Studies with Cows. J. Anim. Sci., 11, (1952): 61.

[11] Kleiber, M., Smith, A. H., and Black, A. L.: Carbonate as Precursor of Milk Constituents in the Intact Dairy Cow. J. Biol. Chem., 195, (1952): 707.

[12] Kochs, W.: Über eine Methode zur Bestimmung der Topographie des Chemismus im thierischen Körper. Arch. ges. Physiol., 20, (1879): 64.

[13] Simphin, T. L., and White, K.: The Formation of Hippuric Acid. The Influence of Benzoate Administration on Tissue Glycine Levels. Biochem. J., 65, (1957): 574.

[14] Snapper, I., Grunbaum, A., and Neuberg, J.: Über die Hippursäuresynthese in der überlebenden Niere von verschiedenen Tiergattungen, auch von Menschen. Biochem. Ztschr., 145, (1924): 40.

[15] Solomon, A. K.: Equations for Tracer Experiments. J. Clin. Invest., 28, (1947): 1927.

SUMMARIO IN INTERLINGUA

Studios del Metabolismo de Glycina-1-C [14] in le Vacca Lactari—Excretion Urinari de Acido Hippuric e Exretion Pulmonar de CO_2

1. Tres vaccas lactari esseva tractate con injectiones intravenose de glycina-1-C^{14}, e le radioactivitate del CO_2 pulmonar e del glycina de acido hippuric in le urina esseva mesurate.

2. Le curvas de tempore del specific activitates standard del CO_2 e del glycina de hippurato es exprimite tanto como excretion cumulative e mathematicamente como le summa de plure componentes del "prime ordine."

3. Duo vaccas lactante e un vacca non lactante habeva expirate, respectivemente, 20,1, 20,1, e 23,5 pro cento del injicite dose de glycina-1-C^{14} in le forma de CO_2 respiratori al fin de un intervallo de trenta-duo horas post de injection.

4. Approximativemente 7 e 9 pro cento, respectivemente, del injicite dose de glycina-C^{14} esseva excernite como glycina de acido hippuric in le urina per 2 vaccas lactante intra le prime 24 horas post le injection. Un non-lactante vacca excerneva approximativemente 5 pro cento del injicite glycina in le forma de acido hippuric intra le prime 24 horas post le injection.

Cyanacethydrazide as a Chemotherapeutic Agent in the Control of Experimental Bovine Verminous Pneumonia

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CHEMOTHERAPEUTIC control of parasitic nematodes living in the air passages of the lungs is a unique problem. The value of an anthelimintic that kills lungworms but does not dislodge them is questionable, since the dead worms, either larval or adult, might serve as foci for pathologic changes. The first encouraging development for treating lungworms was presented by Walley,[12-14] who reported that cyanacethydrazide * would cause the expulsion of live adult lungworms from the air passages of the lungs. He further reported obvious clinical improvement in experimentally infected calves within a few days after treatment. Since 1957, cyanacethydrazide has been employed in numerous field trials and, for the most part, the results have been favorable. O'Donoghue [6] observed that calves, treated twice with cyanacethydrazide, obtained dramatic relief from signs associated with lungworm infection. Trace [11] claimed that cyanacethydrazide-treated cattle had noticeable clinical improvement, and weight gains were significantly greater than in the control animals. Jackson [2] reported cyanacethydrazide was a safe and effective treatment for severe lungworm infection in cattle. He based his opinion on clinical observations, parasitologic studies, and a tabulation of weight gains in 632 lungworm-infected cattle, of which 400 were treated. Stinson [9] reported that cyanacethydrazide was 100 per cent effective in the removal of lungworms from naturally infected cattle.

In a few reports, it has been suggested that cyanacethydrazide therapy has little or no effect on animals with clinical cases of verminous pneumonia. Baxter [1] reported that, on the basis of clinical observations, no distinction could be made between calves treated with cyanacethydrazide and calves left as untreated controls. His observations were on cattle which were naturally exposed and apparently not severely affected, and no attempt was made to ascertain the effect of the drug upon the worm burden in these animals. Swanson [10] reported that cyanacethydrazide treatment was of no value in saving lives or altering the resultant pathologic or clinical changes in naturally or experimentally infected animals.

The experiments reported here were conducted to further ascertain the chemotherapeutic value of cyanacethydrazide against the disease caused by the lungworm of cattle, *Dictyocaulus viviparus*.

MATERIALS AND METHODS

Sixty-seven 4-to-6-month-old Holstein-Friesian calves, purchased at a Fort Collins, Colo., sales yard were used. By fecal examinations made at the time of purchase, the calves were known to be free from adult lungworms; a few were passing occasional trichostrongylid eggs, and about half of them were passing small numbers of coccidial oocysts. The calves were kept in stanchions for a short time prior to and throughout the trials. They were given alfalfa hay, free choice, supplemented with 1 to 2 lb. of a mixed grain concentrate. For each trial, the calves to be used were allotted at random to three groups: (1) an unexposed, unmedicated control group, (2) an exposed, unmedicated group, and (3) an exposed, medicated group.

Dictyocaulus viviparus larvae used in these investigations were initially obtained from Beltsville, Md., and thereafter maintained in either the untreated exposed calves or in others used specifically for this purpose. In the earlier phases of these experiments, larvae were collected from the feces of infected calves and raised to the infective stage as reported by Rubin and Lucker.[8] The number of viable infective larvae recovered by this technique was unreliable. The method described by Jarrett *et al.*[3] was tried and found to be simple yet reliable. According to this method, infected

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* The cyanacethydrazide (Dictycide) used in this work was furnished by Fort Dodge Laboratories, Fort Dodge, Iowa.

feces were put into beakers to a depth of 1 to 2 inches. The beakers were stored in a large covered container, which had about 1 inch of water in the bottom to keep the atmosphere moist. After seven to ten days of storage, the feces were washed with water. These washings contained numerous, vigorous, infective larvae.

Infective larvae were used as soon after collection as possible, usually within one week. Storage during this interval was in a refrigerator. Whenever a group of calves was to be exposed, the available infective larvae were pooled. To determine the total number of larvae present, the number in aliquot samples (0.1 ml.) was counted. For each experimental trial, all exposed calves were given equal numbers of larvae from the same suspension.

Infective larvae, administered in approximately 40 ml. of water, were placed posterior to the base of the tongue by means of a 2-oz. dosing syringe with a 6-inch tube. After each inoculation, the syringe was rinsed with 40 to 50 ml. of water which was also given to the calf.

Daily temperatures and respiration rates and weekly weights were recorded for all calves for one to two weeks before, as well as throughout, the experimental period. To determine the prepatent period for the lungworms in each calf, fecal examinations, using a Baermann funnel, were begun 21 days after exposure and repeated daily until larvae were recovered.

The Effects of Cyanacethydrazide Therapy Given at Different Times to Calves, Following a Single Oral Exposure to 13,250 to 15,000 Infective Larvae. To determine if there was an optimal time to treat an animal in relation to the stage of the lungworm harbored, cyanacethydrazide was administered (1) on postexposure days 14, 15, and 16, (2) on postexposure days 20, 21, and 22 or 21, 22, and 23, and (3) on the fifth, sixth, and seventh days after patency (27 to 33 days after exposure).

Of 44 calves exposed to infection, 21 were treated with cyanacethydrazide given subcutaneously for three consecutive days at the rate of 15 mg. per kilogram of body weight. The injection site was changed daily so that successive injections were not given in the same area. An additional 10 calves were used as unexposed, unmedicated controls.

Each experiment was terminated by necropsy of all but the unexposed calves. The necropsies were usually performed two to three weeks after treatment. Necropsy procedure was routine except for the lungs, and these were examined as described by Rubin and Lucker.[3] The worms recovered by this method were counted individually if the total worms were few, or by a dilution counting technique when they were numerous. The number of worms recovered is presented (table 1).

The Effects of Supportive Medication, with and Without Cyanacethydrazide Therapy, Given to Calves on the Fifth, Sixth, and Seventh Days Following Patency After Exposure to 1,000 Infective Larvae Given Daily for 15 Days.

In an effort to simulate natural exposure conditions, each of 13 calves used in this part of the experiment were exposed to 15 daily doses of 1,000 infective larvae. In addition, in an effort to determine if symptomatic treatment early in the infection had any influence on the course of the infection, the calves involved in this experiment were allotted at random to the following groups: 5 calves were given symptomatic treatment and cyanacethydrazide; 5 calves were given only symptomatic treatment; and the remaining 3 calves served as unmedicated, infected controls.

It was decided to treat the 10 calves in the first two groups at the same time, and to start treatment as soon as any 3 calves out of the 10 had clinical signs of parasitism. Therapy was instituted 14 days after the initial exposure. Symptomatic treatment consisted of four daily i.m. injections of 2,000,000 units of penicillin and 1.25 Gm. of dihydrostreptomycin, as well as an initial oral dose of 480 gr. of sulfathiazole followed by three daily doses of 240 gr. of sulfathiazole.

Calves given cyanacethydrazide were treated at the rate of 15 mg. per kilogram of body weight five, six and seven days after patency. Data collected early in the experiment (table 1) indicated that this was the optimal time for treatment.

Necropsy of this group of calves was performed approximately two weeks following cyanacethydrazide therapy. The number of lungworms recovered from these calves is presented (table 2).

RESULTS

No statistically significant differences could be seen between the numbers of worms recovered from the treated and untreated calves (table 1) because of the wide intra-group variance and, in some instances, the overlapping data between the treated and untreated calves. However, a comparison of the average number of worms recovered from the principals at the different times of treatment with that recovered from the untreated controls for each group justifies the following conclusions: (1) Treatment on postexposure days 14, 15, and 16 is ineffective, as evidenced by the fact that the treated calves had a higher average number of lungworms than did the untreated calves (1,362 *versus* 833). (2) Treatment on postexposure days 20, 21, and 22 was 72.9 per cent effective, with an average of 415 worms per treated and 1,534 per untreated calf. (3) Treatment on postexposure days 21, 22, and 23 was 43.0 per cent effective, with averages per animal of 401 and 704, respectively. (4) Treat-

TABLE 1—A Comparison of the Numbers of Lungworms Recovered at Necropsy from Untreated Calves and from Those Treated with Cyanacethydrazide at Various Times after Exposure

No. of larvae administered	Untreated	Treated on 14, 15 and 16 days after exposure	Untreated	Treated on 20, 21, and 22 days after exposure	Untreated	Treated on 21, 22, and 23 days after exposure	Untreated	Treated between 27th and 33rd days after exposure (5, 6, and 7 days after patency)
	1,489*	1,800	.....	.....	.....	.....	.....	.....
15,000	899	1,843	.....	.....	.....	.....	.....	.....
	111	444	.....	.....	.....	.....	.....	.....
	.....	.....	3,750	680	.....	.....	.....	.....
	.....	.....	840	120	.....	.....	.....	.....
	.....	.....	1,980	320	.....	.....	.....	.....
	.....	.....	560	540	.....	.....	.....	.....
	.....	.....	540	.....	.....	.....	.....	.....
13,250	.....	.....	.....	.....	800	488	.....	.....
	.....	.....	.....	.....	1,199	178	.....	.....
	.....	.....	.....	.....	622	356	.....	.....
	.....	.....	.....	.....	844	29	.....	.....
	.....	.....	.....	.....	55	955	.....	.....
	.....	.....	.....	.....	.....	.....	4,729	15
	.....	.....	.....	.....	.....	.....	166	60
	.....	.....	.....	.....	.....	.....	755	58
	.....	.....	.....	.....	.....	.....	22	259
	.....	.....	.....	.....	.....	.....	110	12
	.....	.....	.....	.....	.....	.....	65	333
	.....	.....	.....	.....	.....	.....	1,000	177
	.....	.....	.....	.....	.....	.....	3,200	196
	.....	.....	.....	.....	.....	.....	20	228
	.....	.....	.....	.....	.....	.....	689	.....
Av. No. of worms recovered per calf	833	1,362	1,534	415	704	401	1,076	149

These data do not include 10 unexposed control calves.
* This and the other figures refer to the number of worms recovered from individual calves.

ment on the fifth, sixth, and seventh days after patency (27 to 33 days after exposure) was 86.1 per cent effective, with averages of 149 and 1,076, respectively.

A comparison (table 2) of the average numbers of worms recovered from (*a*) calves treated symptomatically and with cyanacethydrazide and (*b*) calves treated only symptomatically, shows that the efficacy of cyanacethydrazide was 58.5 per cent, (441 worms *versus* 1,063). These data, however, did not show any statistically significant difference between the two groups. Symptomatic treatment had no observable effect on respiration rates or body temperatures of the treated calves when compared with the untreated calves and in no way seemed to influence the course of the lungworm infection.

The weight gain data are too limited to be useful and are not included in the results. Infected calves, both treated and untreated, were necropsied within two to three weeks after treatment. During this period, no significant differences were observed in the weight changes in the treated calves as compared with those of the untreated calves.

DISCUSSION

On the basis of these studies, the writers are of the opinion that cyanacethydrazide

TABLE 2—A Comparison of the Numbers of Lungworms Recovered at Necropsy from Calves Given No Treatment, Given Symptomatic Treatment, or Given Symptomatic Treatment and Cyanacethydrazide

No. of larvae administered	No cyanacethydrazide, no symptomatic treatment	No cyanacethydrazide, symptomatic treatment	Cyanacethydrazide 5, 6, and 7 days after patency, symptomatic treatment
15,000 (1,000 daily for 15 days)	4,177*	3,400	1,200
	116	1,667	489
	118	229	343
		14	167
		6	8
Average No.	1,470	1,063	441

* This and similar figures refer to the number of worms recovered from individual calves.

is of definite, but limited, value in the treatment of bovine verminous pneumonia. This drug is apparently more efficient in removing mature or nearly mature lungworms than it is in removing the larval forms. However, in the pathogenesis of verminous pneumonia, the larval forms are of considerable importance. During the period of larval development, lung tissue may be irreparably damaged, weight losses are common, and, in some instances, death may occur. Jarrett *et al.*[5] reported that pulmonary lesions are found seven days after exposure, which is two weeks before the worms become adult. In the experiments reported herein, it was observed that cyanacethydrazide is of no value when administered at this stage of the infection. This evidence of the limited usefulness of cyanacethydrazide makes it difficult to understand reports of the remarkable efficacy of the drug in naturally occurring cases.[2,6,9,11]

No immediate clinical improvement in the treated animals could be detected. In addition, there was a wide range in severity of lesions found in the lungs of both the treated and untreated calves at necropsy. However, as mentioned earlier, the pathologic processes are initiated by the larval lungworms, and removel of some or most of these worms when they become fully mature should not be expected to alter the pathologic changes already present. Jarrett *et al.*[5] reached a similar conclusion and stated: "A therapeutic approach to the problem of parasitic bronchitis could not be wholly successful; if a drug were found to kill adult lungworms, the lesions produced by the disease would still remain." In another paper, Jarrett *et al.*[4] offered a more detailed comment concerning the limited value of cyanacethydrazide:

"Three things must be borne in mind, however. One is that calves can die of husk in the prepatent period due to the pulmonary lesions produced by larval migration and it is not to be expected that a drug with a vermifuge action will greatly ameliorate the condition if the infecting dose has been very large; secondly, animals on an infected pasture are picking up larvae until the day treated or removed from that pasture and consequently have 24 days' worth of immature worms still to complete the life cycle. Lastly, if treatment is started during the patent phase when the lesions we have described are well established, one should not expect too rapid a clinical improvement. In fact, under the conditions mentioned above we have been unable to prevent death in several calves using this drug."

The findings of the writers substantiate the observations by Jarrett *et al.*[4] and, in part, those of Swanson.[10] The consensus of investigators appears to be that other agents, chemical or biological, are needed in addition to cyanacethydrazide for the clinical control of lungworm infections in cattle.

In evaluating the efficacy of cyanacethydrazide or any other chemical in this particular host-parasite relationship, the most obvious difficulty is the establishment of uniform infections in experimental animals. It is evident from the data presented that the number of worms that become adult in the lungs cannot be predicted from the number of infective larvae administered. Factors that might cause the failure to obtain uniform infections are (1) a variation in the natural resistance of the host; (2) a variation in the number of larvae that negotiate all of the physical barriers during the process of reaching the lungs following oral exposure; and (3) the development of an acquired host resistance by previous exposure to lungworm larvae. These calves should not have any acquired resistance, because they were all born in an area where bovine verminous pneumonia has not been reported. To further substantiate the fact that these calves had not been previously exposed to lungworm infection, the first larvae always appeared in the feces at the normal interval following experimental exposure. Regardless of the cause, the inability to establish uniform infections made the statistical evaluation of the efficacy of cyanacethydrazide impossible.

Conclusions

Cyanacethydrazide is effective in removing a substantial percentage of adult or nearly adult lungworms *(D. viviparus)* from the lungs of experimentally infected calves. However, no observable benefits were derived from its use early in the course of an infection when the lungworms were immature and were causing severe pathologic changes. Symptomatic treatment early in lungworm infection with penicillin, dihydrostreptomycin, and sulfathiazole is of no apparent value in alleviating clinical signs produced by immature lungworms.

SUMMARY

Forty-four calves were exposed to 13,250 to 15,000 infective *Dictyocaulus viviparus* larvae each. Cyanacethydrazide was administered subcutaneously for three consecutive days at the rate of 15 mg. per kilogram of body weight to 21 of these calves. Treatment was administered to groups of 3 to 10 calves at different times following exposure; there was a comparable number of untreated infected animals for each group. Evaluation of the efficacy of the drug was based on comparisons of the average numbers of worms recovered from the treated and untreated calves at necropsy.

Treatment of 3 calves on postexposure days 14, 15, and 16 was ineffective. The average number of worms from these calves was 1,362, as compared with an average of 833 from 3 control calves. Treatment of 4 calves on postexposure days 20, 21, and 22 was 72.9 per cent effective, based on the average of 415 worms from the treated calves as compared with 1,534 from 5 control calves. Treatment of 5 calves 21, 22, and 23 days after exposure was 43.0 per cent effective, based on the average of 401 worms from the treated calves as compared with 704 from 5 untreated controls. Treatment of 9 calves on the fifth, sixth, and seventh days after patency (27 to 33 days after exposure) was 86.1 per cent effective, based on the average of 149 worms from the treated calves as compared with 1,076 from 10 control calves.

Thirteen additional calves were given 15,000 infective larvae each, at the rate of 1,000 per day for 15 days. Three remained as unmedicated controls; 5 were treated symptomatically; and 5 were treated symptomatically and given cyanacethydrazide on the fifth, sixth, and seventh days after patency. Symptomatic treatment alone did not influence the normal course of the infection. Cyanacethydrazide was 58.5 per cent effective, based on the average of 441 worms from the 5 calves treated both symptomatically and with cyanacethydrazide as compared with 1,063 from the 5 treated symptomatically.

References

[1] Baxter, J. T.: Treatment of Parasitic Bronchitis. Vet. Rec., 69, (1957): 870.

[2] Jackson, R. A.: Losses from Lungworms Stopped by a New Anthelmintic, Dictycide. Calif. Vet., 11, (1958): 20.

[3] Jarrett, W. F. H., McIntyre, W. I. M., and Urquhart, G. M.: Husk in Cattle. Vet. Rec., 66, (1954): 665–676.

[4] Jarrett, W. F. H., McIntyre, W. I. M., Jennings, F. W., and Mulligon, W.: The Natural History of Parasitic Bronchitis with Notes on Prophylaxis and Treatment. Vet. Rec., 69, (1957): 1329–1340.

[5] Jarrett, W. F. H., McIntyre, W. I. M., and Urquhart, G. M.: The Pathology of Experimental Bovine Parasitic Bronchitis. J. Path. & Bact., 73, (1957): 183–193.

[6] O'Donoghue, J. G.: Clinical Trials with Cyanacethydrazide for Treatment of Lungworms in Cattle and Sheep. Canad. J. Comp. Med. & Vet. Sci., 22, (1958): 237–239.

[7] Parker, W. H.: Diethylcarbamazine in the Treatment of Lungworm Infestation of Calves Due to *Dictyocaulus viviparus*. J. Comp. Path. & Therap., 67, (1957): 251–262.

[8] Rubin, R., and Lucker, J. T.: The Course and Pathogenicity of Initial Infections with *Dictyocaulus viviparus*, the Lungworm of Cattle. Am. J. Vet. Res., 17, (April, 1956): 217–226.

[9] Stinson, K. K.: Dictycide Aids Korean Livestock Industry. Biochemic Rev., 28, (1958): 13.

[10] Swanson, L. E., Wade, A. E., Senseman, V. F., and Djfar, M. I.: The Efficacy of Cyanacethydrazide as a Treatment of Lungworm, *Dictyocaulus viviparus* (Bloch), Infections in Cattle. Am. J. Vet. Res., 20, (Sept., 1959): 777–783.

[11] Trace, J. C.: The Diagnosis and Treatment of Lungworm Infection in Cattle. Calif. Vet., 11, (1958): 20.

[12] Walley, J. K.: A New Drug for the Treatment of Lungworms in Domestic Animals, Part I. Vet. Rec., 69, (1957): 815–824.

[13] Walley, J. K.: A New Drug for the Treatment of Lungworms in Domestic Animals, Part II. Vet. Rec., 69, (1957): 850–853.

[14] Walley, J. K.: A New Drug, Cyanacethydrazide, for Oral and Subcutaneous Treatment of Lungworm Disease in Animals. J.A.V.M.A., 131, (Dec. 15, 1957): 539–544.

SUMMARIO IN INTERLINGUA

Cyanacethydrazida Como Agente Chimotherapeutic in le Tractamento de Bovin Pneumonia Verminose de Origine Experimental

Quaranta-quatro vitellos esseva exponite a inter 13.250 e 15.000 larvas infective de *Dictycaulus viviparus* cata un. Cyanacethydrazida esseva administrate per via subcutanee durante tres dies consecutive al rhythmo de 15 mg per kg de peso corporee per die a 21 de iste vitellos. Le tractamento esseva administrate a gruppos de 3 a 10 vitellos a varie intervallos post le exposition. Correspondente gruppos de inficite animales esseva observate sin

medication. Le evalutation del efficacia del droga esseva basate super comparatones del numeros medie de vermes retrovate al necropsia ab le tractate e ab le non-tractate animales.

Le tractamento de 3 vitellos al dies 14, 15, e 16 post le exposition esseva inefficace. Le numero medie de vermes ab iste vitellos esseva 1.362, a comparar con 833 in le caso del 3 vitellos de controlo. Le tractamento de 4 vitellos al dies 20, 21, e 22 post le exposition habeva un efficacia de 72,9 pro cento, a judicar per le retrovation del numero medie de 415 vermes ab iste vitellos in comparation con 1.534 in 5 vitellos de controlo. Le tractamento de 5 vitellos al dies 21, 22, e 23 post le exposition monstrava un efficacia de 43,0 pro cento, con un numero medie de vermes de 401 in comparation con 704 in 5 animales de controlo. Le tractamento de 9 vitellos al dies 5, 6, e 7 post le patentia, i.e. inter 27 e 33 dies post le exposition, habeva un efficacia de 86,1 pro cento, con un numero medie de 149 vermes in le vitellos tractate e 1.076 in 10 vitellos de controlo.

Dece-tres vitellos additional recipeva 15.000 infective larvas cata un, al rhythmo de mille per die durante 15 dies. Tres remaneva sin medication e serviva como animales de controlo; 5 esseva tractate symptomaticamente; e 5 esseva (1) tractate symptomaticamente e (2) injicite con cyanacethydrazida le dies 5, 6, e 7 post le patentia. Le uso de tractamento symptomatic sol non influentiava le curso normal del infection. Cyanacethydrazida habeva un efficacia de 58,5 pro cento, i.e. un numero medie de 441 vermes esseva retrovate in le vitellos tractate dualmente pro symptomas e con cyanacethydrazida, durante que 1.063 esseva le numero correspondente in le 5 vitellos que esseva tractate symptomaticamente sed non con cyanacethydrazida.

The Effect of Dimethoate on *Dermatobia hominis* in Cattle

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THE TROPICAL WARBLE FLY (*Dermatobia hominis*, L., Jr.), also known as the human bot fly, tórsalo, berne, and nuche, is a parasite peculiar to Mexico and Central and South America. It is a common and economically significant parasite of cattle. The larva of this fly can penetrate the skin of most mammals, including man, and there it develops into a warble similar to that of the Hypoderma spp. However, contrary to the Hypoderma spp., no migration takes place. The larval infestation in cattle causes severe damage to hides and may result in reduced weight gains and milk flow. Secondary bacterial infection usually occurs at the site of infestation, and the affected animal becomes unsightly and subject to screwworm attack.

The entire life cycle is completed in about three months, and the larval stage in the mammalian host is about five to seven weeks. Infestations are generally limited to animals at altitudes of 1,000 to 5,000 ft. A short dry season, damp soil, dense cattle population, and potential egg vectors seem to be factors favorable to heavy infestation.[1]

Many methods of controlling Dermatobia infestations have been practiced, including destruction of the larvae by extracting them by hand, keeping the animals away from forests and woods, and by dipping or spraying the animals with insecticidal materials that would inhibit the insect vectors and have residual activity.

From the comparative studies made by Neel *et al.*[4,5] in the vicinity of Turrialba, Costa Rica, it appears that insecticide concentrations of 0.5 per cent toxaphene, or a mixture of 0.5 per cent DDT and 0.03 per cent *gamma* isomer of BHC (lindane), or 0.15 per cent aldrin, when sprayed on cattle at biweekly intervals, gave effective control of *Dermatobia hominis*. There was no significant difference in the degree of control in these tests. Year[7] obtained better results when he combined Smear E.Q. 335 as topical ointment with dips of toxaphene.

A series of experiments was conducted by Graham *et al.*[2] in Nicaragua, using several organic-phosphorus insecticides that had shown promise for the control of *Hypoderma bovis*. Oral doses of dimethoate* proved more effective than oral doses of Bayer 21/199** and ronnel† in the control of *D. hominis* in these trials. Dimethoate also proved effective when administered by injection and as a topical spray.

Dimethoate, known chemically as O,O-dimethyl-S-(N-methylcarbamoylmethyl) phosphorodithioate, has been shown to have effect as a systemic for the control of cattle grubs when administered orally or intramuscularly.[3] Further successful results with dimethoate, obtained during the 1957-1958 warble fly season, encouraged the author to study the insecticide against Dermatobia. Investigations carried out by Peterson *et al.*[6] to evaluate dimethoate for the control of *Oestrus ovis* indicated a high degree of effectiveness against all stages of the larval form of *O. ovis* in sheep given dimethoate. Accordingly, this compound was tested by the author in Costa Rica and Nicaragua in the spring of 1958.

This paper reports the results of these tests against *D. hominis* in cattle, confirming the results of trials undertaken by Graham *et al.*[2]

MATERIALS AND METHODS

A group of 51 Brahma crossbred cattle, weighing 115 to 430 kg., were moderately to severely infested with *D. hominis* larvae when selected. Approximately equal numbers of these cattle were allotted to two trial sights: one near Tilaran, Costa Rica, the other near Matagalpa, Nicaragua. Because of the similarity of cattle and infestations, the data have been combined and are reported as one experiment. Since birth, these cattle had had a history of continuous infestation in various stages. Although some of these cattle had been sprayed with 0.5 per cent chlordan solution

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* Compound Cl 12880, American Cyanamid Co., Princeton, N.J.

** Bayer 21/199 is available from Chemagro Corp., New York, N.Y.

† Trolene, produced by Dow Chemical Co., Midland, Mich.

approximately 22 days prior to the initiation of this experiment, it is believed this did not influence the results of the experiment, as the cattle were heavily and uniformly infested with live Dermatobia at the time of treatment.

Dimethoate was administered either subcutaneously, intramuscularly, or orally. Doses of 10.0, 7.5, 5.0, and 2.5 mg. per kilogram of body weight were administered subcutaneously, and doses of 10.0, 5.0 and 2.5 mg./kg. were given by intramuscular injection. Oral doses were 7.5 mg./kg. Subcutaneous injections were given in the cervical region just in front of the scapula. The intramuscular injections were made in the gluteal region, anterior and lateral to the first coccygeal vertebra. Boluses were administered with a balling gun.

Since it was not feasible to weigh the cattle at the location, estimated weights were used to calculate the dosage levels. The cattle were identified with a paint brand or a permanent tattoo in the left ear.

Following treatment, the cattle were kept in pens for the balance of the day to facilitate close observation. After this, they were turned into a large pasture and observed at frequent intervals for evidence of toxicosis.

Approximately 54 to 96 hours following treatment with dimethoate, the cattle were examined individually and a representative number of larvae was removed surgically or manually. Each larva removed was closely examined to determine whether it was dead or alive. No effort was made to positively determine the larval stage as each larva was removed, but it was noticed that all three larval stages were represented, with those of the second instar clearly in the majority.

RESULTS

The effect of mode of treatment on the per cent of dead larvae is shown (table 1).

TABLE 1—Control of the Larvae of Dermatobia hominis (L., Jr.) by the Subcutaneous, Intramuscular, and Oral Administration of Dimethoate

No. of cattle	Dose (mg./kg.)	Route	Larvae extracted	Larval mortality (%)
6	10.0	s.c.	98	98
9	7.5	s.c.	120	88
7	5.0	s.c.	104	76
6	2.5	s.c.	75	40
5	10.0	i.m.	79	100
7	5.0	i.m.	109	68
5	2.5	i.m.	79	32
6	7.5	oral	59	80

s.c. = subcutaneous; i.m. = intramuscular.

The numbers given in the table represent the number of grubs extracted at the examination following treatment and the per cent of extracted larvae presumably dead due to treatment.

Either intramuscular or subcutaneous injections of 10 mg./kg. results in 98 to 100 per cent mortality of the *D. hominis* larvae examined in the treated cattle. A subcutaneous injection of 7.5 mg./kg. resulted in an average of 88 per cent. Oral treatment with 7.5 mg./kg. resulted in an average mortality of 80 per cent. Doses of 5.0 and 2.5 mg./kg. by subcutaneous injection gave 76 and 40 per cent mortality, respectively. Doses of 5.0 and 2.5 mg./kg. by intramuscular injection gave 68 and 32 per cent, respectively.

Following treatment, 2 of the cattle given 10 mg./kg. intramuscularly developed mild signs of toxicosis (salivation, diarrhea, depression, and inappetence). One animal recovered promptly. The other animal remained slightly depressed and was reluctant to graze for a period of about 18 hours, but this was followed by complete recovery.

The results of this study compare favorably with those obtained by Graham *et al.*[2]

It is the author's opinion that the incidence and degree of toxicosis reported here would not preclude the general use of dimethoate as a means of Dermatobia control.

SUMMARY

Fifty-one *Dermatobia hominis*-infested cattle in Costa Rica and Nicaragua were given dimethoate at several dosage levels by intramuscular, subcutaneous, or oral routes. At varying intervals following treatment, a representative number of larvae was removed from each animal to determine whether they were dead or alive. A dose of 10.0 mg. of dimethoate per kilogram of body weight, given subcutaneously or intramuscularly, resulted in 98 and 100 per cent mortality, respectively, of the larvae examined. Lower doses by these routes and the oral dose of 7.5 mg./kg. resulted in less satisfactory larval mortality.

Two of the cattle given 10 mg./kg. intramuscularly developed signs of toxicosis, but neither the incidence nor severity of these signs was serious enough to preclude the general use of dimethoate for Dermatobia control.

References

[1] Anderson, E. H.: Biology, Distribution and Control of *Dermatobia hominis*. Vet. Med., 55, (1960): 72–78.

[2] Graham, O. H., Kraemer, P., and Osorio, A.: Control del tórsalo (*Dermatobia hominis*, L., Jr.) con insecticidas organicos fosforados, de accion sistemica. Turrialba, 8, (1958): 153–157.

[3] Hewitt, R., Emro, J., Entwistle, J., Pankavich, J.,

Thorson, R., Wallace, W., and Waletzky, E.: Carbamoyl Alkyl Phosphorodithioates as Chemotherapeutic Agents: Effects of Dimethoate Against Grubs in Cattle. J. Econ. Entomol., 51, (1958): 445–450.

[4] Neel, W. W.: Control of Human Bot Fly on Cattle. J. Econ. Entomol., 47, (1954): 540–541.

[5] Neel, W. W., Urbina, O., Hovis, J. R., and de Alba, J.: Combate del tórsalo (*Dermatobia hominis*, L., Jr.) por medio de insecticidas en Turrialba, Costa Rica. Turrialba, 5, (1955): 139–146.

[6] Peterson, H. O., Cobbett, N. G., and Meleny, W. P.: Treatment of *Oestrus ovis* with Dimethoate. Vet. Med., 54, (1959): 377–383.

[7] Year, J. D. E.: Informative Bulletin on Protection of Cattle Against the Human Bot Fly. Ministry of Agriculture and Husbandry, Republic of Nicaragua.

Le Effecto de Dimethoato Super *Dermatobia hominis* in Bestial

Cinquanta-un pecias de bestial in Costa Rica e Nicaragua, omnes infestate de *Dermatobia hominis*, esseva tractate con dimethoato a diverse nivellos de dosage per via intramuscular, subcutanee, o oral. A varie intervallos post le tractamento, un numero representative de larvas esseva prendite ab omne animal pro determinar si illos esseva morte o vive. Un dose de 10,0 mg de dimethoato per kg de peso corporee, administrate subcutanee- o intramuscularmente, resultava in un mortalitate de 98 e 100 pro cento, respectivemente, del larvas examinate. Plus basse doses in administrationes per le mesme vias e le dose oral de 7,5 mg per kg resultava in un minus satisfacente mortalitate larval.

Duo del animales recipiente 10 mg per kg per via intramuscular disveloppava signos de toxicosis, sed ni le severitate ni le frequentia de iste signos esseva satis importante pro arguer contra le uso general de dimethoato in le lucta contra dermatobia.

The Effect of Sodium Selenite on Cattle

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NUMEROUS EXPERIMENTS have been carried out to determine the effects of seleniferous plants and organic and inorganic selenium compounds on laboratory animals. The results obtained by feeding seleniferous plants to livestock and laboratory animals have been attributed solely to selenium.

Seleniferous plants can produce either acute or chronic poisoning in livestock. Chronic selenium poisoning is associated with two well-known diseases, blind staggers and alkali disease. Results of experiments by Draize and Beath [3] indicate that native range plants, such as *Astragalus bisulcatus* and *Xylorrhiza parryi*, containing a moderate or high concentration of water-soluble selenium, can produce the blind staggers type of poisoning.

Forage poisoning, or polioencephalomalacia,[5] a noninfectious disease of pasture and feedlot cattle and sheep, is characterized by multiple foci of necrosis in the cerebral cortex. This disease has been diagnosed in cattle and sheep of Colorado, Wyoming, western Nebraska, and western Kansas.

The clinical syndromes of blind staggers in animals of Wyoming and forage poisoning in animals of Colorado are identical.[5] Research workers in Wyoming are of the opinion that blind staggers in cattle of that state is caused by chronic selenium poisoning.[8] In Colorado, the cause of forage poisoning has not been determined. Unsuccessful attempts have been made to reproduce the disease experimentally by forcibly feeding cattle large amounts of water and plants from areas where the disease developed. Blind staggers has not been reproduced in laboratory animals by feeding diets containing seleniferous plants or sodium selenite.

REVIEW OF LITERATURE

The first authentic written record of selenium poisoning (''alkali disease'') in livestock is the report in 1860 by Madison,[6] an army surgeon stationed at Fort Randall, territory of Nebraska. He described a fatal disease of horses which had grazed in a certain area near the fort. The horses lost the long hair from the mane and tail, and their feet became sore. Soon after people settled around Fort Randall, about 1891, the farmers experienced difficulty with the same disease in their livestock.

Experiments by Franke,[4] in 1929, led to the discovery of selenium as the cause of alkali disease. In 1933, it was discovered that certain native plants growing on seleniferous soils accumulate large quantities of selenium.[4]

Seleniferous plants can produce either acute or chronic poisoning in animals. The toxic effect depends upon the type of selenium compound, the experimental animal used, the conditions of the test, and the criterion of toxicity employed. The selenium compounds which cause alkali disease are found in the proteins of the plants and are relatively insoluble in water, whereas the selenium compounds which produce blind staggers are known to be readily soluble in water.

Smith *et al.*[10] found that the effects of continued small doses of selenium were cumulative, and there was no evidence of acquired tolerance to selenium. Organic selenium was accumulated in higher quantities and was retained longer in the tissues than inorganic selenium.

Alkali disease was produced experimentally with seleniferous barley hay grown in soil that had carried a heavy stand of *Astragalus bisulcatus*.[1] The hay which had an average selenium content of 25 parts per million was fed green to 2 yearling calves for 46 days. At the end of four weeks, their joints became stiff and their hoofs cracked horizontally and vertically. By the end of four months, the hoofs of both animals were badly affected and had started to slough.

Alkali disease was induced in hogs in controlled feeding experiments.[7] Wheat from affected farms produced alkali disease in 14 days. The hair of the hogs began to fall out, and, within a short time, most of then were hairless.

Schwarz *et al.*[9] recently identified selenium as the integral part of factor III. Factor III prevents liver necrosis in the rat, multiple necrotic degeneration in the mouse, and exudative diathesis in the chick. It was inferred that selenium is an essential trace element.

The purpose of the following experiments was to determine the effect of sodium selenite, an inorganic selenium compound, on cattle under typical feedlot conditions.

From the College of Veterinary Medicine and the Colorado Agricultural Experiment Station, Colorado State University, Fort Collins. Dr. Clopton is professor of chemistry, University of Colorado, Boulder.

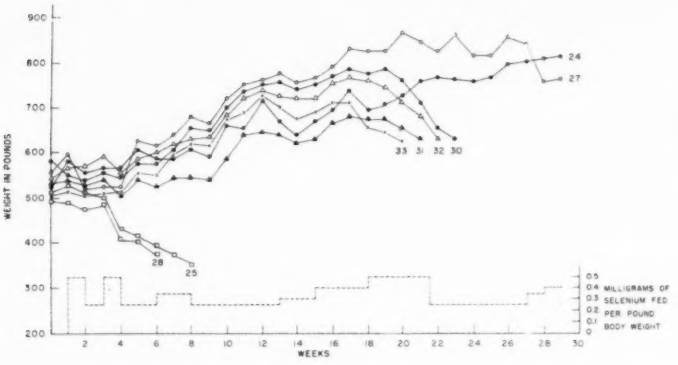

Fig. 1—Growth curves of steers fed a normal ration plus sodium selenite. The broken line indicates the amount of selenium, as sodum selenite, fed each week.

MATERIALS AND METHODS

Thirteen Hereford steers of uniform size and averaging 541 lb. were acquired from commercial sources. They were reported to have come from near Dallas, Texas.

The 13 steers were allotted at random to two lots. Five steers, designated as controls, were given a ration consisting of ground grain, alfalfa hay, and molasses. The ground grain portion of the ration was composed of a mixture containing one third corn, one third milo, and one third barley; molasses in the ration was 8 per cent by weight. The remaining 8 steers, the principals, were fed the same ration plus known quantities of sodium selenite.

Representative samples of all feed and water which the experimental cattle consumed were analyzed for possible selenium content. Weights were recorded, urine samples were collected, and blood samples were taken from each steer at weekly intervals. Three times each week, the principals were fed sodium selenite. The solution of sodium selenite was placed in 1½-oz. gelatin capsules and the capsules were administered by means of a balling gun. This method made it possible to know the exact number of milligrams of selenium consumed by each steer. The amount of selenium administered was based on body weight, insuring that each steer was given the same relative amount of selenium.

A necropsy was performed on each animal. The selenium content of samples of hair, horns, muscle, liver, kidneys, spleen, and brain was determined. The feet were removed and placed in a freezer for future comparison.

Cheng[9] developed a spectrophotometric method for the determination of traces of selenium. This method was modified for use with plant and biological substances by freeing the selenium from the organic material by digestion. The digestion procedure followed in this experiment was the same as that described by Trelease and Beath[11]

RESULTS

No selenium was fed during the first week of the experiment. During the second

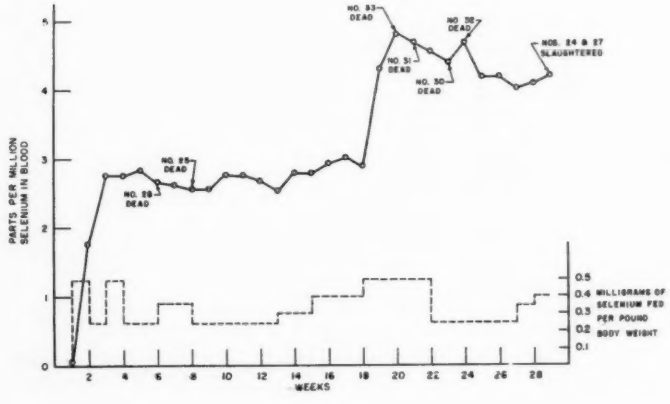

Fig. 2—Average selenium content in blood of steers fed a normal ration plus sodium selenite. The broken line indicates the quantity of selenium fed.

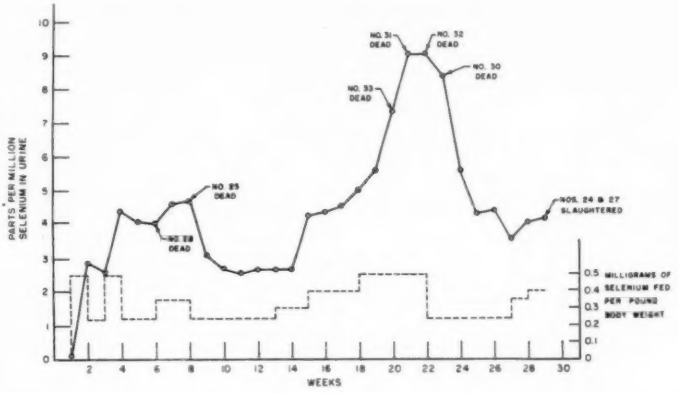

Fig. 3—Average selenium content in urine of steers fed a normal ration plus sodium selenite. The broken line indicates the quantity of selenium fed.

week, each of the principals was fed 0.50 mg. of selenium (as sodium selenite) per pound of body weight. After two feedings at that level, all 8 principals were inappetent and appeared depressed.

The dosage of sodium selenite was decreased to 0.25 mg. of selenium per pound of body weight during the third week of the experiment. At this level, all visible signs of toxicosis disappeared.

During the fourth week of the experiment, the concentration of the selenium was restored to 0.50 mg. per pound of body weight. This level caused anexoria as before, indicating that 0.50 mg. of selenium per pound of body weight was more than the steers could tolerate.

From the end of the fourth week to the end of the eighteenth week, the amount of selenium fed varied between 0.25 mg. and 0.40 mg. per pound of body weight. Two of the principals died within eight weeks. After 18 weeks, no visual signs of chronic poisoning were observed in any of the remaining principals. They ate their rations each day and gained weight. At the beginning of the nineteenth week, the selenium dosage was increased to 0.50 mg. per pound of body weight. The first administration at this level produced inappetence. The maintenance of this increased dosage resulted in the death of 4 of the steers (fig. 1). These steers lost considerable weight, but did not develop weakness until the terminal stages.

The average selenium content of the blood of the principals at weekly intervals is shown (fig. 2). As long as the selenium content in the blood remained at 3.0 p.p.m. or less, there were no signs of poisoning. Severe toxicosis occurred in the steers when the selenium content of the blood became greater than 3.0 p.p.m. When the blood selenium content became greater than 4.0 p.p.m., the animals ceased eating and became depressed and inactive.

The average selenium content in the urine of the treated steers is also shown (fig. 3).

While moving and handling the animals, a peculiar trembling of skeletal muscles was noticed in the principals. Exercise accentuated this trembling which in some cases was so marked that the animals were shivering. This condition was not observed in the controls.

The 6 steers that died during the experiment were found to have severe gastrointestinal irritation at necropsy. Rumenitis, commonly seen in cattle on a fattening grain ration, was more accentuated and prolonged in the principals than in the controls. All of the principals had abomasitis, varying from shallow ulceration of the mucosa to gangrene as was seen in animal number 31. Enterocolitis in the principals was characterized by hyperemia and catarrhal inflammation. At necropsy, 2 steers (25 and 30) had lesions of polioencephalomalacia characterized by considerable neuronal degeneration of the cerebral cortex and less pronounced neuronal degeneration in the cerebellar cortex. No other changes attributable to selenium were noticed, except a pronounced tubular nephrosis in animal 32.

Signs of central nervous system involvement in steer 25 were weakness and inabil-

TABLE 1—Selenium Content in Tissues of Experimental Cattle

Steer No.	Brain	Hair	Horns	Kidneys	Liver	Muscle	Spleen
SELENIUM-TREATED STEERS							
24	0.58	9.58	4.80	3.21	5.10	0.64	2.41
25	0.19	3.93	0.29	3.05	5.00	0.15	1.05
27	0.60	9.80	4.41	3.21	6.33	0.62	2.94
28	0.10	1.41	0.20	3.40	6.00	0.10	1.05
30	0.70	9.56	.....	2.55	7.80	0.39	3.24
31	0.58	11.12	.....	2.86	12.30	0.68	2.12
32	0.54	11.25	2.24	4.37	7.50	0.73	3.16
33	0.40	10.92	1.52	4.34	10.12	0.10	2.32
CONTROL STEERS							
21	0.05	1.22	.....	1.22	1.14	0.09	0.40
22	0.16	1.24	1.10	1.12	1.20	0.07	0.32
23	0.12	1.32	0.80	1.32	1.15	0.11	0.40
26	0.10	0.94	.....	1.10	1.13	0.09	0.43
29	0.15	1.26	0.94	1.08	1.15	0.10	0.38

Selenium contents are given in parts per million.

ity to rise. In 24 hours, this condition gradually progressed to a state of coma. Steer 30 was in a state of aggressive frenzy and incoordination for approximately one hour. The following hour he struggled violently but was unable to rise. After 48 hours of coma, euthanasia and necropsy were performed. Steers 24 and 27, killed at the termination of the experiment, had no relevant tissue lesions.

No detectable amount of selenium was found in the water, ground grain, or molasses consumed by the cattle. The alfalfa hay contained 0.11 p.p.m. of selenium.

The amounts of selenium found in the organs and tissues of the animals examined are presented (table 1). The tissues, in descending order of selenium concentration, were: hair, liver, kidneys, spleen, muscle, and brain.

At the termination of the experiment, several veterinary pathologists examined the feet of all 13 steers; and they were unable to distinguish between the feet of the steers given selenium and those of the controls.

DISCUSSION

In this work, the cattle fed selenium developed few signs typical of alkali disease, blind staggers, or forage poisoning.

Previous experiments, designed to produce chronic poisoning of the alkali disease type in cattle, have not been successful. The general signs of alkali disease include dullness, emaciation, lameness, anemia, roughened coats, loss of long hair, and lesions and deformities of the hoofs.

The growth curve (fig. 1) and the average selenium blood level curve (fig. 2) indicate that the consumption of selenium at subtoxic levels does not produce a cumulative effect, and that an animal can tolerate some selenium without ill effects. The recent work of Schwarz[9] has shown that animals require trace amounts of selenium in the diet.

Studies have been made of the effects of feeding seleniferous plants to livestock and laboratory animals. The ill effects produced have been attributed solely to the presence of selenium in the plants. Alkaloids and other toxic substances have been found in many of the seleniferous plants, which may either singly or collectively cause the syndromes commonly attributed to selenium poisoning. There are striking differences in the effects produced by feeding inorganic selenium compounds and by feeding seleniferous plants to livestock. In this experiment, feeding sodium selenite for 28 weeks did not produce hoof deformities, roughened coats, shedding of the long hair, or other classic signs of the alkali disease syndrome.

SUMMARY

Thirteen steers of uniform size and weight were allotted at random to two lots. Eight steers were fed a ration of ground grain, alfalfa hay, and molasses plus quantities of selenium (as sodium selenite) based on body weight. The other 5 steers were fed the same ration without selenium.

Selenium was given orally three times a week. The amounts varied from 0.25 mg. to 0.50 mg. per pound of body weight. Two of the steers died within eight weeks. Four more died after 20 to 23 weeks.

In this experiment, cumulative effects from sodium selenite were not demonstrated. It was shown that there is a level of

dosage of sodium selenite which an animal can tolerate without visible ill effects.

Acute sodium selenite toxicosis was manifested by severe gastrointestinal irritation, inappetence, and depression, leading finally to incoordination, coma, and death. In 2 of the steers, histologic lesions of polioencephalomalacia were present.

References

[1] Beath, O. A., Eppson, H. F., and Gilbert, C. S.: Selenium and Other Toxic Minerals in Soils and Vegetation. Wyoming Agric. Exper. Sta. Bull. 206, 1935.

[2] Cheng, K. L.: Determination of Traces of Selenium. Anal. Chem., 28, (1956): 1738.

[3] Draize, J. H., and Beath, O. A.: Observations on the Pathology of "Blind Staggers" and "Alkali Disease." J.A.V.M.A., 86, (June, 1935): 753–763.

[4] Franke, G. W.: A Toxicant Occurring Naturally in Certain Samples of Plant Foodstuffs. I. Results Obtained in Preliminary Feeding Trials. J. Nutrition, 8, (1934): 596–608.

[5] Jensen, Rue, Griner, L. A., and Adams, O. R.: Polioencephalomalacia of Cattle and Sheep. J.A.V.M.A., 129, (Oct. 1, 1956): 311–321.

[6] Madison, T. C.: Sanitary Report—Fort Randall. Statistical Report on the Sickness and Mortality in the Army of the United States. 36th U.S. Congr., 1st Sess., Senate Ex. Doc., 52, (1860): 37.

[7] Moxon, A. L.: Alkali Disease or Selenium Poisoning. South Dakota Agric. Exper. Sta. Bull. 311, (1937): 1–91.

[8] Rosenfeld, I., and Beath, O. A.: The Pathology of Selenium Poisoning. Wyoming Agric. Exper. Sta. Bull. 275, (1946): 1–27.

[9] Schwarz, Klaus, and Foltz, C. M.: Selenium as an Integral Part of Factor 3 Against Dietary Necrotic Liver Degeneration. J. Am. Chem. Soc., 79, (1957): 3292.

[10] Smith, M. I., Stohlman, E. F., and Lillie, R. D.: The Toxicity and Pathology of Selenium. J. Pharmacol. & Exptl. Therap., 61, (1937): 89–102.

[11] Trelease, S. F., and Beath, O. A.: Selenium. Published by the authors, New York, (1949): 253–264.

SUMMARIO IN INTERLINGUA

Le Effecto de Selenito de Natrium in Bestial

Dece-tres tauros de peso e dimensiones uniforme esseva dividite al hasardo in duo gruppos. Octo recipeva un dieta de grano molite, feno de lucerna, e melassa e in plus selenium in le forma de selenito de natrium in quantitates basate super le peso corporee. Le altere 5 tauros recipeva le mesme dieta sin selenium.

Le selenium esseva administrate tres vices per septimana per via oral. Le quantitates variava inter 0,25 e 0,50 mg per libra de peso corporee. Duo del tauros moriva intra octo septimanas. Quatro alteres moriva post inter 20 e 23 septimanas.

In iste experimento, effectos cumulative de selenito de natrium non esseva demonstrate. Esseva trovate que il existe un limine de dosage de selenito de natrium que un animal pote tolerar sin visibile effectos adverse.

Acute toxicosis per selenito de natrium esseva manifeste per sever irritation gastrointestinal, inappetentia, e depression, resultante ultimemente in incoordination, coma, e morte. In 2 del tauros, lesiones histologic de polio-encephalomalacia esseva presente.

Anthelmintics for Ruminants. I. Studies on the Toxicity and Efficacy of Bayer 21/199 as an Anthelmintic

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DUE TO INTEREST in the possible use of certain organic phosphorus compounds as anthelmintics and to the promising results obtained in a preliminary trial of one such compound by the authors,[1] it seemed advisable to collect more information on the toxicity and efficacy of this compound, Bayer 21/199 [0,0-diethyl 0-(3-chloro-4 methyl-7-coumarinyl) phosphorothioate],* in calves and lambs. This compound, used by McGregor *et al.*[4] in an effort to find a compound with systemic insecticidal properties, has been recommended to control certain arthropod parasites of cattle and sheep.[8]

Herlich and Porter[3] reported a high degree of anthelmintic efficiency by this compound against species of the genera Haemonchus, Ostertagia, Trichostrongylus, Cooperia, Strongyloides, Trichuris, and Oesophagostomum in lambs and calves when given at doses of 25 mg. per kilogram of body weight. In most cases, lower doses had the same or slightly lower efficiency. In previous work, however, the authors[1] have shown that, in some cases at least, the compound produces fatal intoxication in lambs at the 25 mg./kg. level. Gordon[2] reported that some fatalities occurred in sheep given doses of 7.7 mg./kg. Stampa,[7] in treating sheep with this compound, reported that the lowest lethal dose was 17.6 mg./kg. Of 400 sheep given this dose, 2 died.

Radeleff[6] reported that a dose of 50 mg./kg. resulted in the death of 1 heifer, while a dose of 25 mg./kg. produced no signs of toxicity in another heifer. Herlich and Porter[3] reported none or only mild and transient signs of toxicosis in calves given doses of 25 mg./kg.

From the Department of Veterinary Parasitology, School of Veterinary Medicine, and the Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station, where Dr. Galvin is an instructor, Dr. Turk is professor and head, and Dr. Bell is associate professor.

* Available from the Chemagro Corp., New York, N.Y.

MATERIALS AND METHODS

Two trials were run with Bayer 21/199.

Trial 1 was a preliminary check to determine the general anthelmintic effect. Therefore, only 3 lambs were used. Two were given 12.5 mg./kg. as a suspension, while the third lamb served as a control. Post-treatment worm counts were made by a method described under trial 2.

Trial 2 was made with 11 lambs and 7 calves. The lambs had been kept on pasture for about three months when placed in a barn on a concrete floor a few days before treatment. The calves had been on pasture for several months when confined in a small lot for treatment and observation.

The calves were used for toxicity studies. For that purpose, they were allotted so that 1 calf was given 50.0 mg./kg., 1 calf was given 37.5 mg./kg., 2 calves were given 25.0 mg./kg., 2 calves were given 12.5 mg./kg., and 1 calf served as a control. The calves were dosed orally with the powder in gelatin capsules.

The lambs were allotted into groups whereby 2 lambs were given 12 mg./kg., 3 lambs were given 6 mg./kg., 3 lambs were given 3 mg./kg., and 3 lambs served as controls. The lambs were given the compound as a drench after mixing it with water and bentonite.

In addition to gross observations, toxicity of the compound for the treated animals in trial 2 was studied by means of a kidney function test (blood creatinine levels) and whole blood cholinesterase activity determinations. The latter was determined by a modification of Michel's method.[5] Instead of washed erythrocytes, 0.4 ml. of whole blood was mixed with 9.6 ml. of saponin. Michel's red blood cell buffer was used. Cholinesterase activity is expressed as change in ΔpH units per hour. A blank was run with each set of blood samples to determine nonenzymatic hydrolysis of acetylcholine instead of using the f and b factors utilized by Michel. Pretreatment and post-treatment hemoglobin and packed blood cell determinations were made on each animal in conjunction with the cholinesterase activity determination (table 2).

Anthelmintic effect of Bayer 21/199 in the lambs was assessed at necropsy by counting the various kinds and numbers of worms present in the gastrointestinal tracts of the principals as compared to those present in the control animals.

Worm counts were made by washing the abomasum, small intestine, and large intestine separately and taking aliquot samples. The aliquots

TABLE 1—Doses and Worm Counts in Lambs in Trial 1

Lamb No.	Treatment	Weight (kg.)	Dose (mg./kg.)	Abomasum			Small intestine			Large intestine
				Trich.	Haem.	Ost.	Trich.	Coop.	Nem.	(Oesoph.)
14	Control	....	.....	420	360	270	2,846	321	1,423	72
15	21/199	24	12.5	0	0	30	150	0	150	0
16	21/199	23	12.5	60	0	60	60	0	210	108

Trich. = Trichostrongylus spp.; Haem. = Haemonchus spp.; Ost. = Ostertagia spp.; Coop. = Cooperia spp.; Nem. = Nematodirus spp.; Oesoph. = Oesophagostomum spp.

from the abomasums and small intestines were examined under a dissecting microscope. The parasites were picked out and either all or the first 100 were identified as to genus. The aliquots from the large intestines were examined grossly, and the parasites were picked out and identified by genus. If more than 100 nematodes were found in an aliquot, only the first 100 were identified and the percentage of each genus therein was determined. By multiplying the total number of each genus in the aliquot by the dilution, the total number of each genus was determined.

RESULTS

Trial 1.—Post-treatment worm counts from the lambs used in trial 1 are given (table 1). Although the number of lambs used was small, the results indicate a high degree of activity against Haemonchus spp. and Cooperia spp., and some activity against Trichostrongylus spp., Ostertagia spp., and Nematodirus spp. Lamb 15 had a slight salivation from 11 to 48 hours post-treatment, but none at later observations. There were no signs of toxicity in lamb 16.

Trial 2.—In the second trial, the worm counts from lambs (table 3) again indicated a high activity against Haemonchus spp., particularly at the 12 mg./kg. dosage. Cooperia spp. were very susceptible at all dosages used. Although Trichostrongylus spp. were markedly affected as related to percentage reduction, a large number remained, particularly in the small intestine. There was little or no activity against other genera of gastrointestinal parasites.

None of the lambs in this trial had signs of toxicity. However, the calves were markedly affected.

Calf 253 had a transient diarrhea with some blood for 2.5 days after treatment.

Calf 335 had a transient diarrhea two days after treatment. On the fifth and sixth days following treatment, a slight salivation was noticed.

Calf 215 was weak and emaciated prior to treatment. A diarrhea commenced on the day following treatment and became progressively worse until the calf died, six days following treatment. Some blood was passed in the feces on the third day after treatment. Salivation began two days after treatment and was a constant sign until death, at which time the ground under the

TABLE 2—Dosage Levels, Packed Blood Cell Volumes and Hemoglobin Determinations in Lambs and Calves in Trial 2

Animal No.	Weight (kg.)	Dose (mg./kg.)	Pretreatment		Post-treatment*	
			Hemoglobin	Hematocrit	Hemoglobin	Hematocrit
Lamb 148	23	12.0	6.9	27	8.3	31
Lamb 132	27	12.0	7.7	28	8.2	31
Lamb 106	34	6.0	8.7	26	9.6	33
Lamb 105	30	6.0	7.4	28	....**	....**
Lamb 103	23	6.0	6.2	23	7.4	30
Lamb 116	21	3.0	6.8	21	7.5	31
Lamb 141	22	3.0	4.0	17	5.6	22
Lamb 130	25	3.0	10.7	31	8.5	31
Lamb 127	30	Control	6.1	25	4.9	20
Lamb 101	25	Control	8.4	25	6.7	25
Lamb 104	21	Control	4.9	16	5.4	18
Calf 265	164	50.0	5.2	19	7.9	32
Calf 224	164	37.5	10.5	28	6.5†	26
Calf 336	125	25.0	12.0	40	8.3	34
Calf 215	157	25.0	13.7	42	....**	....**
Calf 253	152	12.5	10.7	44	10.2	37
Calf 335	143	12.5	12.1	41	9.7	35
Calf 252	.....	Control	13.0	44	10.2	44

* Blood was collected from sheep eight days after treatment, and from calves nine days after treatment.
** No sample. † Blood was collected seven days after treatment.

TABLE 3—Worm Counts from Gastrointestinal Tracts of Lambs in Trial 2

Lamb No.	Dose (mg./kg.)	Abomasum				Small intestine					Large intestine	
		Haem.	Ost.	Trich.	U.L.	Strong.	Trich.	Coop.	Nem.	Mon.*	Trich.	Oesoph.
148	12	30	0	0	0	60	1,740	30	30	....**	0	0
132	12	30	60	0	0	93	4,557	0	0	....**	0	0
106	6	150	30	450	30	30	2,820	0	90	....**	0	12
105	6	30	0	0	0	0	3,840	0	0	120	0	0
103†	6	720	210	300	60	336	3,750	0	84	930	0	0
116	3	330	0	120	0	300	1,440	0	60	330	0	12
141	3	450	180	480	90	814	6,300	0	296	450	0	24
130	3	900	0	1,230	120	90	360	0	0	30	0	0
127	Cont.	11,810	0	544	1,496	400	7,760	1,500	100	690	84	12
101	Cont.	7,053	0	1,278	255	466	5,728	400	0	870	0	0
104	Cont.	7,495	0	2,707	208	844	14,188	1,858	0	690	48	0

Haem. = Haemonchus spp.; Ost. = Ostertagia spp.; Trich. = Trichostrongylus spp.; U.L. = Unidentified larvae; Strong. = Strongyloides spp.; Coop. = Cooperia spp.; Nem. = Nematodirus spp.; Mon. = Moniezia spp.; Oesoph. = Oesophagostomum spp.

* Most Moniezia spp. consisted of immature specimens with only the scolex and a few immature proglottids present. ** Not counted. † Also had 42 Bunostomum spp.

calf's head was saturated with saliva. A discharge from the eyes became apparent two and one half days following treatment and was a constant sign until death. On the fifth day following treatment the calf was down with its head extended and was unable to rise.

salivation was noticed on the fifth day following treatment. The calf died 15 days after treatment from peritonitis resulting from other causes.

Calf 265 had a diarrhea beginning on the second day following treatment; the feces contained blood and mucus. Tenesmus

TABLE 4—Whole Blood Cholinesterase Activity in Calves in Trial 2 Before and Following Treatment with Bayer 21/199

Calf No.	Normal*	Dose (mg./kg.)	Hours post-treatment						Days post-treatment								
			1	2	4	8	12	24	2	3	4	5	7	9	11	15	18
215	21.5	25.0	19	20	21	17	9	7	4	5	4	2	4**	....	....	....	....
224	23.0	10.5	31	30	32	27	25	11	10	7	7	3	4	7	10	10	9
253	40.0	12.5	41	34	47	40	41	23	20	19	18	20	20	20	23	27	27
265	21.0	50.0	21	25	27	17	9	9	8	8	7	1	4	....	....	....	....
335	31.0	12.5	27	31	29	28	24	17	16	16	14	10	15	16	19	23	18
336	22.5	25.0	23	18	22	19	11	7	6	6	8	3	5	7	7	9	10
252	33.0	Control	35	31	31	35	33	39	35	36	33	33	31	37	39	37	38

* Average of two pretreatment samples. ** Six days; sample taken at time of death.

Calf 336 had a diarrhea first noticed seven hours after treatment and lasting until about five days after treatment. Some blood and mucus were observed in the feces on the day following treatment.

Calf 224 had a constant diarrhea beginning on the day after treatment. A slight

was seen on the third day. Small amounts of diarrheal feces, usually blood-tinged, were passed until the calf died on the eighth day following treatment. Slight salivation began two days after treatment and was noticed periodically until the death of the calf, at which time the salivation had be-

TABLE 5—Whole Blood Cholinesterase Activity in Lambs in Trial 2 Before and Following Treatment with Bayer 21/199

Lamb No.	Normal*	Dose (mg./kg.)	Hours post-treatment						Days post-treatment					
			1	2	4	8	12	24	2	3	4	5	7	8
148	13.5	12	12	12	10	7	9	5	5	6	7	5	9	11
132	11.5	12	11	13	10	10	8	6	3	7	6	6	8	10
106	11.5	6	10	12	8	9	9	8	9	8	10	6	8	10
105	13.0	6	10	13	9	11	10	8	7	9	11	6	10	11
103	10.0	6	9	10	7	9	9	7	7	8	9	4	8	12
116	14.5	3	9	14	11	10	13	12	12	11	12	11	14	16
141	11.0	3	10	9	11	10	9	9	9	10	10	9	12	13
130	15.0	3	11	9	12	14	13	13	14	12	13	11	12	15
127	12.5	Control	11	11	11	13	12	12	11	11	11	10	9	12
101	14.0	Control	12	14	14	15	14	15	13	12	13	12	10	15
104	9.5	Control	10	10	8	8	9	9	9	9	9	7	10	9

* Average of two pretreatment samples.

come profuse. After the fifth day following treatment, the calf was incoordinated and had difficulty in rising.

These observations are in marked contrast to those reported previously,[1] in which no signs of toxicosis were seen in calves given 25 mg./kg. of body weight.

Blood creatinine levels were determined to check on possible damage to the kidneys. Determinations were made on a pretreatment sample from each animal and on samples collected four, 12, 24, 48, 72, 120, and 192 hours following treatment. No significant change was noticed, with the exception of calf 265 which had a pretreatment and 120-hour level of 1.4 mg. per 100 ml. of blood and a level of 2.1 mg. at the 192-hour check.

Whole blood cholinesterase activities were markedly reduced in all calves (table 4), regardless of the dose.

Reduction of cholinesterase activity in lambs (table 5) seemed to have a more direct relationship to the dosage level than was observed in the calves. In those lambs in which a significant decrease in activity occurred, the change usually began two to four hours following treatment.

The calves which were given 12.5 to 50.0 mg. per kilogram of body weight developed marked signs of toxicosis. Two deaths occurred at the higher dosage levels.

There was no apparent correlation between kidney function tests and signs of toxicosis in either lambs or calves. In calves, the changes in whole blood cholinesterase activity following treatment had no relationship to the dosage within the scope of this experiment. In lambs, there seemed to be a direct relationship of cholinesterase depression to the dosage level.

Summary

Bayer 21/199 was tested for anthelmintic activity and toxicity in lambs, and for toxicity in calves. The compound was very active against Haemonchus spp. and Cooperia spp., but less so against other genera at the dosages used (3 to 12.5 mg./kg. of body weight). Gross signs of toxicosis were noticed in 1 lamb.

References

[1] Galvin, T. J., Bell, R. R., and Turk, R. D.: The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as Anthelmintics in Ruminants. Am. J. Vet. Res., 20, (Sept., 1959): 784–786.

[2] Gordon, H. McL.: Studies on Anthelmintics for Sheep. Some Organic Phosphorus Compounds. Austral. Vet. J., 34, (1958): 104.

[3] Herlich, H., and Porter, D. A.: An Anthelmintic for Cattle and Sheep. Critical Tests of Efficacy of Bayer 21/199. Vet. Med., 53, (1958): 343.

[4] McGregor, W. S., Radeleff, R. D., and Bushland, R. C.: Some Phosphorus Compounds as Systemic Insecticides Against Cattle Grubs. J. Econ. Entomol., 47, (1954): 465–467.

[5] Michel, H. O.: An Electrometric Method for the Determination of Red Blood Cell and Plasma Cholinesterase Activity. J. Lab. & Clin. Med., 34, (1949): 1564.

[6] Radeleff, R. D., Woodard, G. T., Nickerson, W. J., and Bushland, R. C.: The Acute Toxicity of Chlorinated Hydrocarbon and Organic Phosphorus Insecticides to Livestock. U.S.D.A. Tech. Bull. 1122, (1955): 46 pp.

[7] Stampa, S.: The Control of Internal Parasites of Sheep with Neguvon and Asuntol. J. South African Vet. Med. A., 30 (1959): 19.

[8] U.S. Department of Agriculture: Insecticide Recommendations of the Entomology Research Division for the Control of Insects Attacking Crops and Livestock 1959 Season. Agriculture Handbook No. 120, Rev., 1959.

Summario in Interlingua

Anthelminticos pro Ruminantes. I. Studios del Toxicitate e Efficacia de Bayer 21/199 Como Agente Anthelmintic

Bayer 21/199 esseva testate pro su activitate anthelmintic e pro su toxicitate in agnos e pro su toxicitate in vitellos. Le composito esseva activissime contra Haemonchos spp. e Cooperia spp. Su activitate esseva minus marcate contra altere generes al dosages usate in le tests, i.e. a inter 3 e 12 mg per kg de peso corporee. Grossier signos de toxicitate esseva notate in 1 agno.

Le vitellos que esseva tractate con inter 12,5 e 50,0 mg de Bayer 21/199 per kg de peso corporee disveloppava marcate signos de toxicosis. Duo mortes occurreva al plus alte de iste nivellos de dosage.

Esseva constatate nulle correlation inter le tests de function renal e le signos de toxicosis in le agnos e nulle in le vitellos. In vitellos, le alterationes del activitate sanguinee de cholinesterase total post le tractamento non revelava un relation con le dosages usate intra le limites del experimento. In agnos, il pareva exister un relation directe inter le depression de cholinesterase e le nivello de dosage.

Anthelmintics for Ruminants. II. Anthelmintic Activity and Toxicity of Ruelene in Sheep

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SEVERAL OF THE organic phosphates have been reported as having anthelmintic activity against nematode parasites of ruminants.[2, 4-7] Ruelene (4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate)* has been reported to have anthelmintic activity against ruminant parasites as evaluated by egg counts per gram of feces. Doses of 300 mg. or more per kilogram of body weight caused signs of intoxication in some sheep.[1]

A series of trials was set up to determine further this compound's activity against various genera of gastrointestinal parasites of sheep and to establish a safe, effective dose.

MATERIALS AND METHODS

Trial 1 was directed toward determining general anthelmintic effect and finding a satisfactory dosage level for use in another trial with a greater number of lambs. Eight lambs were used in this trial. Two lambs were given 400 mg./kg. of body weight, 1 was given 200 mg./kg., 2 were given 100 mg./kg., and 3 lambs served as controls. Post-treatment worm counts were made on all lambs (table 1).

Kidney function tests were made by checking blood creatinine levels before treatment and at 26 and 67 hours thereafter. Whole blood cholinesterase activity was determined prior to treatment and at intervals of four, 18, and 26 hours and two, three, five, six, seven, eight, nine, and 12 days following treatment.

Trial 2 was run primarily to determine further the toxic effects of this compound. Of the 4 lambs that were used, 2 were given 200 mg./kg. of body weight and 2 were given 400 mg./kg. In order to detect possible effects on liver function, the bromsulfophthalein test was performed before treatment and on the third day following treatment. Whole blood cholinesterase activity was determined prior to treatment and at various intervals thereafter until this activity had returned to the pretreatment level (table 3). Post-treatment packed cell volume and hemoglobin determinations were made (table 2).

In trial 3, 18 lambs were used. Twelve were given 100 mg./kg. and 6 served as controls. Whole blood cholinesterase activity was checked prior to treatment and at intervals of one, two, four, eight, 12, and 24 hours and two, three, six, and seven days following treatment.

The lambs in trials 1 and 3 were killed for necropsy seven to 13 days following treatment, and worm counts were made by a dilution method described in a previous paper.[3] Whole blood cholinesterase activities were determined by the electrometric method previously described.[3]

TABLE 1—Dosage Rates and Worm Counts on Lambs Used in Trial 1

Lamb No.	Weight (kg.)	Dose (mg./kg.)	Abomasum			Small intestine					Large intestine (Trichuris)
			Haem.	Ost.	Trich.	Coop.	Trich.	Strong.	Nem.	Mon.	
567	24	Control	0	0	3,360	0*	5,491	125	437	0	24
594	28	Control	0	0	120	0	2,745	175	0	0	....†
82	....**	Control	6,984	176	1,940	93	3,441	325	511	1	60
72	23	100	30	0	0	0	60	30	0	1	24
59	27	100	30	0	1,980	0	2,644	335	441	0	0
77	30	200	30	0	60	0	90	0	0	0	36
554	28	400	0	0	0	0	0	0	0	1	12
76	22	400	0	0	0	0	0	60	0	0	0

Haem. = Haemonchus spp. Ost. = Ostertagia spp. Trich. = Trichostrongylus spp. Coop. = Cooperia spp. Strong. = Strongyloides spp. Nem. = Nematodirus spp. Mon. = Moniezia spp.
* Also had 187 unidentified larvae. ** Not weighed. † Not examined.

TABLE 2—Dosage Rates and Packed Blood Cell Volume and Hemoglobin Determinations on Lambs Used in Trial 2

Lamb No.	Dose (mg./kg.)	Weight (kg.)	Packed blood cell volume	Hemoglobin
121	200	17.7	31	10.5
107	200	18.1	32	8.4
149	400	18.6	35	8.9
110	400	18.6	38	11.0

From the Department of Veterinary Parasitology, School of Veterinary Medicine, and the Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station, where Dr. Galvin is an instructor, Dr. Bell is associate professor, and Dr. Turk is professor and head.

* Available from the Dow Chemical Co., Midland, Mich.

TABLE 3—Whole Blood Cholinesterase Activity Pretreatment and Post-Treatment Levels Expressed as Change in △ ph Units Per Hour

Sheep No.	Dosage (mg./kg.)	Normal	Hours post-treatment				Days post-treatment															
			1	2	4	8	1	2	3	4	5	8	9	10	11	14	15	16	17	18	25	43	46
121	200	0.16	0.08	0.07	0.07	0.06	0.08	0.08	0.09	0.09	0.08	0.09	0.10	0.09	0.10	0.11	0.11	0.09	0.11	-0.12	0.12	0.17	0.15
107	200	0.15	0.14	0.09	0.08	0.07	0.07	0.08	0.08	0.10	0.10	0.11	0.12	0.11	0.13	0.13	0.14	0.13	0.15	0.14	0.13	0.17	0.16
149	400	0.12	0.07	0.05	0.07	0.06	0.06	0.06	0.05	0.07	0.05	0.06	0.07	0.06	0.09	0.09	0.07	0.09	0.11	0.07	0.06	0.12	0.13
110	400	0.12	0.07	0.07	0.07	0.06	0.04	0.05	0.07	0.08	0.07	0.06	0.06	0.08	0.08	0.08	0.08	0.09	0.09	0.06	0.07	0.15	0.12

*Normal cholinesterase activity was determined by checking a blood sample taken prior to treatment.

RESULTS

Trial 1.—From worm counts, it was seen that there might have been some degree of anthelmintic activity against several genera of parasites present in the gastrointestinal tract. However, since the worm burdens of the controls were so variable, it is difficult to evaluate accurately the results of the treatment against certain genera (table 1).

Gross signs of intoxication were noticed in the lambs given doses of 200 and 400 mg./kg. of body weight. Lamb 76 was severely affected and had excessive salivation and incoordination within four hours after treatment. Four hours later, these signs were markedly reduced. Lambs 77 and 554 were noticeably salivating eight hours following treatment. All lambs were apparently normal when checked 18 hours following treatment.

The blood creatinine levels were unaffected. In contrast, the whole blood cholinesterase activity was markedly affected, particularly at the higher doses. Four hours following treatment, this activity was depressed 0, 46, 73, 85, and 89 per cent of the pretreatment levels in lambs 72, 59, 77, 554, and 76, respectively. The low point in activity was observed at 18 hours following treatment, at which time the depression of activity was 60, 73, 64, 100, and 100 per cent, respectively. Later, there was a progressive rise in activity until the lambs were killed for necropsy 12 days following treatment, at which time the activity was depressed 0, 9, 46, 62, and 56 per cent, respectively.

Trial 2.—The bromsulfophthalein liver function test showed no significant changes. However, only one post-treatment check was made.

A slight salivation occurred in lambs 107, 149, and 110 four hours following treatment. Four hours later, these lambs were apparently normal. Lamb 121 was not visibly affected.

Whole blood cholinesterase activity dropped one hour after treatment (table 3). The degree of depression was similar at both dosage levels (200 and 400 mg./kg.). Maximum depression was reached in two to 23 hours after treatment. A progressive return to pretreatment levels began one to five days following treatment. Whole blood cholinesterase activity in lamb 107 had returned to normal by the seventeenth day following treatment;

TABLE 4—Dosage Rates and Worm Counts on Lambs Used in Trial 3

Lamb No.	Weight (kg.)	Dose (mg./kg.)	Abomasum				Small intestine						Large intestine	
			Haem.	Trich.	Ost.	U.L.	Coop.	Strong.	Trich.	Nem.	Mon.	U.L.	Trichuris	Oes.
112	22	100	150	120	0	30	270	210	840	0	0	0	0	0
113	23	100	930	1,560	0	60	363	242	11,515	0	0	0	24	0
123	18	100	90	30	0	0	30	240	240	30	0	0	0	0
133	17	100	960	540	30	30	43	2,981	1,210	43	0	43	24	0
109	31	100	150	30	0	0	390	1,005	8,385	0	0	0	12	0
145	20	100	870	420	60	0	0	420	2,310	0	0	30	0	24
136	21	100	0	1,260	0	30	330	516	5,874	0	30	0	48	12
144	22	100	0	0	0	0	0	210	300	0	0	0	0	0
117	24	100	0	90	0	0	390	180	1,080	30	0	0	0	0
138	27	100	210	30	0	0	30	270	810	30	0	0	0	0
125	20	100	180	60	0	30	0	540	570	0	30	0	0	0
119	27	100	0	0	0	0	30	60	90	60	0	0	0	0
150	19	Control	1,440	30	0	240	497	621	2,981	0	0	0	24	0
114	24	Control	570	120	0	0	150	630	1,140	30	0	240	12	0
147	26	Control	1,350	600	0	0	630	360	1,020	0	30	30	24	0
129	29	Control	1,350	930	0	0	1,339	223	5,655	0	0	223	84	0
124	15	Control	4,057	773	0	0	1,728	576	26,208	0	30	288	780	0
142	23	Control	150	120	0	0	240	30	420	0	0	60	12	0

Haem. = Haemonchus; Trich. = Trichostrongylus; Ost. = Ostertagia; U.L. = Unidentified larvae; Coop. = Cooperia; Strong. = Strongyloides; Nem. = Nematodirus; Mon. = Moniezia; Oes. = Oesophagostomum.

whereas, the activity in the other 3 lambs did not return to pretreatment levels until sometime between 25 and 43 days after treatment.

Trial 3.—No signs of intoxication occurred in lambs in trial 3. Whole blood cholinesterase activity was depressed from 0 to a maximum of 50 per cent of pretreatment levels. The depression of activity ranged from 23 to 29 per cent in 8 of these lambs. Activity had returned to pretreatment levels in all animals, with the exception of 1 lamb which still had 6 per cent depression seven days following treatment.

Worm counts from these lambs (table 4) show that, although several genera of parasites were markedly reduced in numbers as compared to the controls, large numbers of parasites were not removed.

SUMMARY

Three trials, involving 30 lambs, were made with Ruelene to determine its anthelmintic properties and toxic effects. A comparison of the worm counts of untreated control lambs with the post-treatment worm counts of treated lambs shows that at doses of 100 mg. per kilogram of body weight too many parasites were left in the gastrointestinal tract to recommend Ruelene for use in mixed infections. The higher doses may have more activity; however, signs of intoxication appeared in lambs given 200 mg./kg. or more of the compound.

Whole blood cholinesterase activity was depressed to a maximum of 50 per cent at the 100 mg./kg. dose and was markedly depressed at higher doses.

Liver function tests were run on 4 lambs and blood creatinine determinations were made on 5. There were no significant deviations from normal.

References

[1] Dow Chemical Company: Ruelene, A New Anthelmintic and Systemic Insecticide. ACD Information Bulletin 115, Midland, Mich., May, 1959.

[2] Galvin, T. J., Bell, R. R., and Turk, R. D.: The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as Anthelmintics in Ruminants. Am. J. Vet. Res., 20, (Sept., 1959): 784–786.

[3] Galvin, T. J., Turk, R. D., and Bell, R. R.: Anthelmintics for Ruminants. I. Studies on the Toxicity and Efficacy of Bayer 21/199 as an Anthelmintic. Am. J. Vet. Res., 21, (Nov., 1960): 1054–1057.

[4] Gordon, H. McL.: Studies on Anthelmintics for Sheep. Some Organic Phosphorus Compounds. Austral. Vet. J., 34, (1958): 104.

[5] Herlich, H., and Porter, D. A.: An Anthelmintic for Cattle and Sheep. Critical Tests of Efficacy of Bayer 21/199. Vet. Med., 53, (1958): 343.

[6] Riek, R. F., and Keith, R. K.: Studies on Anthelmintics for Cattle. IV. The Organic Phosphorus Compound O, O-Dimethyl 2, 2, 2-Trichloro-1-Hydroxymethyl Phosphonate (Bayer L 13/59). Austral. Vet. J., 34, (1958): 93.

[7] Stampa, S.: The Control of Internal Parasites of Sheep with Neguvon and Asuntol. J. South African Vet. Med. A., 30, (1959): 19.

SUMMARIO IN INTERLINGUA

Anthelminticos pro Ruminantes. II. Activitate Anthelmintic e Toxicitate de Ruelena in Oves

Tres experimentos—utilisante un total de 30 oves—esseva effectuate con Ruelena pro determinar su proprietates anthelmintic e su effectos toxic. Un comparation del numeration de vermes in non-tractate agnos de controlo con le numeration de vermes in le agnos tractate monstra que con le dosage de 100 mg per kg de peso corporee le supervivente parasitos in le vias gastro-intestinal es troppo numerose pro recommendar Ruelena como medication in infectiones mixte. Plus alte doses es possibilemente plus active, sed signos de intoxication appareva in agnos recipiente 200 mg o plus per kg de peso corporee.

Le nivello del activitate de cholinesterase total in le sanguine esseva deprimite per usque maximalmente 50 pro cento al nivello de dosage de 100 mg per kg de peso corporee. A plus alte doses de Ruelena, le activitate de cholinesterase esseva deprimite ancora plus mareatemente.

Tests del function hepatic esseva executate in 4 agnos; determinationes del creatinina del sanguine in 5. In iste parametros, nulle significative deviationes ab le norma esseva constatate.

Propagation and Titration of Vaccinia Virus in Bovine Epidermal Cells

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SUCCESSFUL PROPAGATION of vaccinia virus in tissue culture [3, 4, 6, 8, 10, 12, 14-20] suggested the use of a monolayer of bovine embryonic cells (BEC) for growing vaccinia virus. This procedure would free the supernatant fluid of the heterologous protein of a plasma clot and the excess homologous protein of the tissue pieces in the Maitland culture as used by Wesslen.[20]

This is a report of propagation and titration of vaccinia virus in monolayer cultures of BEC and use of this virus as a vaccine in rabbits.

MATERIALS AND METHODS

Host Cells.—A bovine embryo within the uterus, obtained from a local abattoir, was brought to the laboratory within a few hours after slaughter of the pregnant cow. When needed, amniotic fluid was collected aseptically through puncture of the amniotic membrane. Embryos 35 to 40 cm. in length were considered satisfactory for use. Skin was stripped from the embryo, washed thoroughly with Hanks' balanced salt solution,[7] minced with scissors, and treated with 1:250 trypsin (Difco) in a final concentration of 0.25 per cent. Gentle agitation with a magnetic stirrer for 20 minutes liberated cells from the skin. After centrifugation at 800 r.p.m. for 10 minutes, the cells (at a 1:400 dilution) were suspended in a growth medium consisting of 70 per cent bovine amniotic fluid, 20 per cent horse serum, and 10 per cent bovine embryo extract. Other mediums, containing 10 to 20 per cent horse serum in medium 199 [9] or Eagle's basal medium [5] without bovine embryo extract, were found satisfactory. To the growth mediums, 50 μg. of dihydrostreptomycin per milliliter and 100 units of penicillin per milliliter were added. Once established, BEC cell lines were serially passaged with suspensions containing 80,000 to 100,000 cells per milliliter. Sheets of cells covered the glass surface within five to seven days when incubated at 37 C.

Titration of Virus.—For virus titration, monolayers were established in test tubes and culture fluids were replaced with a maintenance medium consisting of medium 199, containing 2 to 5 per cent horse serum. Five to six tubes of BEC cultures were inoculated with 0.1 ml. of tenfold serial dilutions of a vaccinia virus suspension and were incubated in a stationary position at 37 C. for seven days, with a change of maintenance medium after the third or fourth day. Seven days after inoculation, cultures were examined for characteristic cytopathic effects and 50 per cent endpoints were calculated by the method of Reed and Muench.[13] Titers were expressed as number of 50 per cent tissue culture infectious doses ($t.c.i.d._{50}$) per milliliter or original virus suspension. Titrations were also performed on rabbits whose skins had been scarified.[1]

Virus.—The vaccinia virus used in these experiments was a dermatropic strain utilized in a commercially available immunizing preparation. The vehicle for the virus was calf lymph. This virus is hereafter designated as VVCL.

RESULTS

Morphologically, young cultures of BEC appeared to be epithelial-like cells with rather large nuclei. After three days' incubation of virus and tissue, cytopathic changes characterized by a rounding-up of a few localized cells were apparent. This rounding-up of cells progressively involved

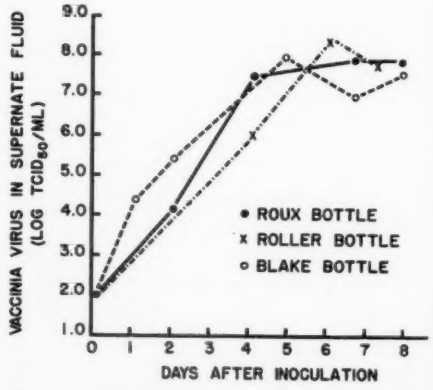

Fig. 1—Time-yield curve of vaccinia virus propagated at 37 C. in stationary and roller bottles with bovine embryonic skin cultures.

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TABLE 1—Titration of VVCL Vaccine Lot 1186 in Cultures of Bovine Embryonic Cells

BEC * Passage No.	Titer of VVCL vaccine 1186 (log t.c.i.d.$_{50}$/ml.)
Primary	7.88, 8.00, 7.94, 8.00, 7.64, 7.96, 7.54, 7.73, 7.94, 8.20 (G.M., 7.88) **
4	8.00
7	7.85
9	7.94, 8.10
14	7.88
15	7.40, 7.51
22	7.70
28	7.84
32	7.40, 7.94
35	8.10

* BEC = bovine embryonic cells. ** G.M. = geometric mean.

more and more cells, until 90 to 100 per cent of the cells had become detached from the glass by the seventh day of incubation. In stained cover slip preparations of these infected cells, the elementary bodies characteristic of vaccinia virus were seen.

When rabbit skin was infected with vaccinia virus derived from calf lymph or tissue culture, the characteristic "take" developed in three to four days. Inclusion bodies were observed in histologic sections of the rabbit skin "takes."

To produce large quantities of virus, monolayer cultures were propagated in a growth medium consisting of Eagle's basal medium and 20 per cent horse serum. Following incubation at 37 C. for five to seven days, the cell cultures were washed three times with medium 199 to remove the serum; fresh medium 199, in the absence of serum, was added before infection with virus. Stationary cultures were prepared in 1-liter Roux, 1-liter Blake, and 2- and 5-liter Povitsky bottles. A culture in a roller bottle was also established. Each of these cultures was inoculated with 10 to 100 t.c.i.d.$_{50}$/ml. of a virus seed. Cultures were incubated at 37 C. for seven days, during which time samples of supernatant fluid were removed for titration. Supernatant fluid in all cultures yielded 10^7 to 10^8 t.c.i.d.$_{50}$/ml. of virus in five to seven days, regardless of whether the cultures were rotated or stationary (fig. 1).

Titrations were performed ten times within one year with VVCL vaccine (lot 1186) to determine the reproducibility of titers obtained in monolayers of primary BEC, which were between 7.54 and 8.54 t.c.i.d.$_{50}$/ml. with a geometric mean of 7.88 (table 1). Virus titers obtained in serially passaged cells were of the same order as those obtained in primary BEC, indicating no alteration in sensitivity of the cells upon passage.

Potency of VVCL vaccine was determined by simultaneous titrations in BEC and on rabbit skin. Although the number of trials was small, it is apparent that the titers obtained in tissue culture were uniform (table 2), while response of rabbit skin varied greatly.

A series of titrations of vaccinia virus in calf lymph and of the same strain of virus propagated in BEC were conducted on scarified skins of rabbits to determine the comparative infectivity of the agent in these vehicles. A standardized VVCL vaccine (lot 1190) was titrated simultaneously with the tissue culture virus on individual animals. Tested in BEC, both preparations had infectivity titers of approximately 10^8 t.c.i.d.$_{50}$/ml. By comparative titration studies (table 3) it was found that tissue-culture propagated virus could meet the potency requirements of the National Institutes of Health for a smallpox vaccine.[1]

To ascertain whether alteration in the protective antigenic character of the virus occurs in BEC, 2 rabbits were vaccinated with VVCL vaccine and 2 with first passage vaccinia virus grown in primary cultures of BEC. A fifth rabbit was used as a control. Vaccination was carried out by the multiple puncture method.[11] All inoculated rabbits developed the characteristic "take." Fifty-seven days postvaccination, each rabbit was challenged with 0.03 ml. of 10^8 t.c.i.d.$_{50}$ VVCL on one side of the shaved back and with a similar amount of tissue culture virus on the other side of the back. Challenge inoculum was administered in the same fashion as the vaccine. Practically all the erythema which was mechanically induced disappeared within 24 to 30 hours. On the third day, rabbit 20 (nonimmune animal) developed an area

TABLE 2—Titration of VVCL Vaccine Lot 1186 in Bovine Embryonic Cells and on Scarified Skins of Rabbits

Trial	BEC titration end points (log t.c.i.d.$_{50}$/ml.)	Dilution of virus applied to scarified skins of rabbits * 1:1,000	1:3,000	1:10,000	1:30,000
1	7.88	90**	50(2)	33(1)	25(2)
2	8.00	95	90(1)	66	(1)
3	7.94	(7)	(3)	(1)	(0)
4	8.00	100	80(3)	50(5)	(12)
5	7.64	75(1)	50(10)	50(3)	(1)

* 0.2-ml. inoculum per site. ** Percentage of scarified area covered by confluent eruption. Numbers in parentheses represent number of discrete lesions.

TABLE 3—Comparative Titrations of Vaccinia Virus Prepared in Calf Lymph and in Tissue Culture

Side of rabbit	Preparation	Infectivity titer t.c.i.d.$_{50}$ /ml. in BEC *	Dilution of virus (rabbit scarification) 1:1,000	1:3,000	1:10,000	1:30,000
R	CL 1190	$10^{8.2}$	100**	100	100	60
L	TC 204	$10^{7.5}$	100	100	80	70
R	CL 1190	$10^{8.2}$	100	100	90	(2)
L	TC 204	$10^{7.5}$	(3)	(2)	0	0
R	CL 1190	$10^{8.2}$	100	100	100	100
L	TC 203	$10^{8.6}$	100	100	100	100
R	CL 1190	$10^{8.2}$	100	100	100	40
L	TC 203	$10^{8.6}$	50	60	80	(2)
R	CL 1190	$10^{8.2}$	100	100	70	5(2)
L	TC 206	$10^{8.5}$	80	(2)	10	0
R	CL 1190	$10^{8.2}$	100	50	40	(3)
L	TC 206	$10^{8.5}$	(5)	(4)	(5)	(2)
R	CL 1190	$10^{8.2}$	100	100	100	40(5)
L	TC 206	$10^{8.5}$	5(6)	(3)	(1)	0
R	CL 1190	$10^{8.2}$	100	90	70	(10)
L	TC 206	$10^{8.5}$	30	90	(1)	0
R	CL 1190	$10^{8.2}$	100	100	100	5(5)
L	TC 206	$10^{8.5}$	100	100	100	50
R	CL 1190	$10^{8.2}$	100	70	25	(2)
L	TC 206	$10^{8.5}$	0	0	0	0
R	CL 1190	$10^{8.2}$	80	5(1)	0	0
L	TC 206	$10^{8.5}$	0	0	0	0

* BEC = bovine embryonic cells. ** Percentage of scarified area covered by confluent eruption. The numbers in parentheses represent the number of discrete lesions.

TABLE 4—Cross-Challenge Experiment in Rabbits Vaccinated with Tissue Culture Vaccine or VVCL Vaccine

Rabbit No.	Immunizing vaccine	Vaccine potency (log t.c.i.d.$_{50}$ /ml.)	Protection against challenge * VVCL vaccine	Tissue culture vaccine
61	calf lymph	8	+	+
55	calf lymph	7	±	±
68	tissue culture	8	+	+
60	tissue culture	7	+	+
20	none	....	−	−

* Challenge was made with 0.03 ml. of 10^8 t.c.i.d.$_{50}$ per milliliter. − = no protection, typical lesion; ± = partial protection; + = complete protection, no lesion.

of erythema with induration which progressed to pustulation by the fifth to sixth day. Rabbit 55, the partially immune animal, responded with some erythema and induration without pustulation, and in the completely immune animals (60, 61, 68) there was no response other than that mechanically induced by the challenge inoculation (table 4).

DISCUSSION

The current practice in evaluating potency of smallpox vaccine is the rabbit scarification technique, as set forth by the N.I.H. minimum requirements.[1] In view of wide variation of response in the rabbit, it is necessary to titrate a vaccine of unknown potency and a vaccine of accepted potency in parallel and in the same animal. Another method employed to evaluate potency is the counting of pox on the chorioallantoic membrane of embryonating chicken eggs.[11] An egg method, proposed by Cabasso,[2] is based on lethality of vaccinia virus for the embryonating egg.

In studies of the propagation of vaccinia virus in monolayer tissue cultures of BEC, it was found that this virus can be readily propagated in this system; titers of 10^7 to 10^8 t.c.i.d.$_{50}$/ml. have been obtained. The simplicity of the tissue culture system as compared to rabbit scarification or egg titration makes it a desirable assay procedure for vaccinia virus, especially in view of the excellent reproducibility of titers obtained.

Using vaccinia virus propagated in tissue cultures of BEC, the present authors obtained typical "takes" in rabbits. Time of appearance of the early papule and evolution of the "takes" were comparable to those that followed successful administration of VVCL vaccine. In addition, on examination of histologic sections of the rabbit skin lesions, typical vaccinial inclusion bodies were seen.

In preliminary experiments it was found that tissue culture virus elicited a strong immune response in rabbits. On the basis of these observations, further studies on the antigenic properties of vaccinia virus derived from tissue culture are in progress.

SUMMARY

Propagation and titration of vaccinia virus in monolayer tissue cultures of bovine epidermal cells were studied. Using this system, methods of preparing vaccinia virus in titers of 10^7 to 10^8 t.c.i.d.$_{50}$/ml. are described. An evaluation of this tissue culture procedure for titration of vaccinia virus indicated that this system was a highly reproducible and sensitive assay system. Evidence that a tissue culture vaccinia virus is immunogenic in the rabbit was presented.

References

[1] Anon.: Minimum Requirements, Smallpox Vaccine. National Institutes of Health, Bethesda, Md. 3rd rev., 1951.

[2] Cabasso, V. J., and Moore, I. F.: A Proposed Potency Test for Smallpox Vaccine. I. Evaluation of Chick Embryo LD_{50} Method. Proc. Soc. Exptl. Biol. & Med., 95, (1957): 605.

[3] Cracium, E. C., and Oppenheimer, E. H.: Vaccinia-Virus in Tissue Culture. Johns Hopkins Medical Research Club, 37, (1925): 428.

[4] Cutchins, E., and Warren, J.: Comparative Susceptibility of Cell Cultures to Vaccinia Virus: Application to the Standardization of Smallpox Vaccine. Proc. Soc. Exptl. Biol. & Med., 97, (1958): 456.

[5] Eagle, H.: Nutrition Needs of Mammalian Cells in Tissue Culture. Science, 122, (1955): 501.

[6] Frenkel, H. S., and Kapsenberg, J. G.: The Growth of Vaccinia Virus on Explanted Skin Tissue of Beef and Sheep Fetus. Nederl. tijdschr. geneesk., 98, (1954): 991.

[7] Hanks, J. H., and Wallace, R. E.: Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration. Proc. Soc. Exptl. Biol. & Med., 71, (1949): 196.

[8] Maitland, H. B., and Maitland, M. C.: Cultivation of Vaccinia Virus without Tissue Culture. Lancet, 2, (1928): 596.

[9] Morgan, J. F., and Morton, H. J.: Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. Proc. Soc. Exptl. Biol. & Med., 73, (1950): 1.

[10] Muckenfuss, R. S., and Rivers, T. M.: Survival of Vaccinia Virus Separated from Living Host Cells by Collodion Membranes. J. Exptl. Med., 51, (1930): 149.

[11] Overman, J. R., and Tamm, I.: Quantitative Titration of Vaccinia Virus on the Chorioallantoic Membrane. J. Immunol., 76, (1956): 228.

[12] Parker, F., Jr., and Nye, R. N.: Studies on Filterable Viruses. Am. J. Path., 1, (1925): 325.

[13] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hyg., 27, (1938): 493.

[14] Rivers, T. M.: Cultivation of Vaccine Virus for Jennerian Prophylaxis in Man. J. Exptl. Med., 54, (1931): 453.

[15] Rivers, T. M., Haagen, E., and Muckenfuss, R. S.: Observations Concerning the Persistence of Living Cells in Maitland's Medium for the Cultivation of Vaccine Virus. J. Exptl. Med., 50, (1929): 181.

[16] Rivers, T. M., and Ward, S. M.: Further Observations on the Cultivation of Vaccinia Virus for Jennerian Prophylaxis in Man. J. Exptl. Med., 58, (1933): 635.

[17] Sanders, M., Kiem, I., and Lagunoff, D.: Cultivation of Viruses. Arch. Path., 56, (1953): 148.

[18] Steinhardt, E., Israeli, C., and Lambert, R. A.: Studies on the Cultivation of the Virus of Vaccinia. J. Infect. Dis., 13, (1913): 294.

[19] Warren, J., and Cutchins, E. C.: General Characteristics and Viral Susceptibility of Bovine Embryonic Tissue Cultures. Virology, 4, (1957): 297.

[20] Wesslen, T.: The Production of Smallpox Vaccine in Tissue Culture of Bovine Embryonic Skin. Arch. ges. Virusforsch., 5, (1956): 430.

SUMMARIO IN INTERLINGUA

Le Propagation e le Titration de Virus de Vaccinia in Bovin Cellulas Epidermic

Le propagation e titration de virus de vaccinia esseva studiate in histoculturas monostratal de bovin cellulas epidermic. Le titros esseva exprimite como le numero del doses inficiente 50 pro cento del histocultura per millilitro del suspension original de virus. Es describite methodos pro preparar, con le uso de iste systema, titros de virus de vaccinia de 10^7 a 10^8. Un evalutation de iste technica histocultural pro le titration de virus de vaccinia indicava que le systema es altemente reproducibile e sensibile como systema de essayage. Es presentate datos que indica que virus de vaccinia de origine histocultural es immunogene in le conilio.

The Effect of Enzyme Inhibitors on *Vibrio fetus*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*

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BOVINE VIBRIOSIS is generally recognized as one of the specific infectious diseases affecting reproduction in cattle. In spite of considerable effort by many workers, reliable, simple, diagnostic methods remain to be developed. Demonstration of *Vibrio fetus* by culture technique, the present method, is greatly hampered by the rather slow growth of *V. fetus* in conventional mediums and consistent contamination by fast-growing saprophytic bacteria of samples to be tested. The significance of contaminating bacteria has been emphasized by several authors.[24,28,59,69] *Proteus vulgaris* and *Pseudomonas aeruginosa*, probably the most common inhabitants of the prepuce of the bull and the external genitalia of females, have caused the most concern.[24,28,50,69] The problem of restriction of the contaminants in the original inoculums has been attacked along several lines. Dilution and distribution of the inoculum has been used by several workers.[12,28,38,50,51] Isolation by infecting laboratory animals represents another approach.[1-3,22,39,53,57] The first attempts to restrict the growth of contaminants in mediums inoculated with material to be examined for *V. fetus* were made by Ryff and Lee.[57] They reported that incorporation of crystal violet or brilliant green in concentrations 1:500,000 and 1:100,000, respectively, retarded the growth of *Escherichia coli* and streptococci but not that of one ovine strain of *V. fetus*. Terpstra and Eisma[69] tested the effect of various substances on the growth of *V. fetus* in blood agar medium. Growth was inhibited by methyl violet, crystal violet, gentian violet, thionin, malachite green, trypoflavine, bismuth nitrate, sodium benzolenite, potassium tellurite, and thallium acetate. Brilliant green (1:500,000) and bile (1:75) permitted growth of *V. fetus*. Results of continued work by the Dutch workers[70] indicated that alkyl-aryl sulfonate depressed certain gram-positive bacteria in concentrations harmless to *V. fetus*. Proteus and Pseudomonas organisms were not affected. Esculin (Merck) in a 1:200 dilution restricted the swarming of Proteus cultures, while no effect was observed on *V. fetus* and Pseudomonas organisms. Cohen[15] tested acetic acid, penicillin, dihydrostreptomycin, bacitracin, polymyxin B sulfate, neomycin, chlortetracycline, and oxytetracycline in regard to their activity on *V. fetus* and bacterial contaminants and found that *V. fetus* did not have any selective resistance to these substances. Unrestricted growth of *P. vulgaris* and *Ps. aeruginosa* in medium containing 0.15 per cent ricinoleate and 0.27 per cent Esculin (Merck) was reported by Steger.[65] Kuzdas and Morse[37] proposed a more complex selective medium containing ethyl violet, bacitracin, polymyxin B sulfate, and Actidione.* They reported inhibition of most bacterial contaminants, except species of Proteus and Pseudomonas.

Marked differences in growth rates of *V. fetus*, *P. vulgaris*, and *Ps. aeruginosa*, as well as differences in their fermentation of carbohydrates, have been observed. This suggests an existence of different metabolic pathways and, consequently, a possibility of depressing or blocking the essential metabolism of one or both of the contaminants without a significant adverse effect on *V. fetus*. The present study was designed to explore this possibility, comparing the sensitivity of *V. fetus* with that of

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* Produced by the Upjohn Co., Kalamazoo, Mich.

Ps. aeruginosa and *P. vulgaris* to various enzyme inhibitors.

MATERIALS AND METHODS

Cultures.—Twenty-two bovine strains of *V. fetus* were employed in this study. Fifteen had been isolated from aborted fetuses; seven, from semen. All strains were catalase-positive. The four strains each of *Ps. aeruginosa* and *P. vulgaris* employed in this investigation were isolated in this laboratory from semen and preputial fluid. The stock cultures were maintained in 125 by 15-mm screw-capped culture tubes containing approximately 10 ml. of the basal medium.

Basal Medium.—Six medium bases and several agar concentrations were compared in a preliminary study in order to obtain a basal medium supporting a consistent and relatively fast growth rate of *V. fetus*. Brucella broth (Albimi) containing 0.1 per cent agar was selected to be the basal medium and henceforth will be referred to as the basal medium.

TABLE 1—Grading Scheme Used in Recording Observations of Bacterial Growth

Numerical values	Description of growth
0	No growth.
1	Barely perceptible density.
2	Distinct density. Thickness of growth zone less than 1 mm.
3	Distinct density. Thickness of growth zone 1 mm.
4	Marked density. Thickness of growth zone 1 to 2 mm.
5	Heavy density. Thickness of growth zone 2 to 3 mm.
6	Heavy density. Thickness of growth zone 2 to 3 mm.
7	Heavy density. Thickness of growth zone 3 to 4 mm.
8	Heavy density. Thickness of growth zone 4 to 5 mm.
9	Heavy density. Thickness of growth zone 5 to 10 mm.
10	Heavy density. Thickness of growth zone 7 to 10 mm.

Enzyme Inhibitors.—The following 19 enzyme inhibitors, varying in mode and site of effect, were selected for this experiment: sodium azide; sodium fluoride; semicarbazide; hydroxylamine nitrate; mercuric chloride; p-chlormercuribenzoate; copper sulfate; sodium arsenite; iodoacetic acid; iodosobenzoic acid; 2,4-dinitrophenol; disodium ethylenediamine tetra-acetate (EDTA); 2,6-diaminopurine; malonic acid; isonicotinic acid hydrazide; N-ethylmaleimide; alkyldimethylbenzylammonium chlorides; triethanolamine-duodecylbenzyl sulfonate (TEADA); and 2,3,5-triphenyltetrazolium chloride.

Inoculum.—Inoculums were prepared from 48-hour cultures of *V. fetus* and 24-hour cultures of *Ps. aeruginosa* and *P. vulgaris*. Inoculums were standardized against the suspended culture of *V. fetus* having the least vigorous growth. All the remaining cultures to be used in the experiment were diluted with sterile basal medium in order to obtain equal density. Five drops, amounting to approximately 0.07 ml. of the suspensions, was employed as the inoculum.

Experimental Mediums.—The mode of incorporation of enyme inhibitors in the mediums was adapted to the solubility and heat stability of the substance to be tested. The pH of test mediums was adjusted to 7. All treated mediums were tested for sterility by 48 hours of incubation prior to inoculation.

Inoculation and Incubation.—Every strain was inoculated into two sets of test tubes. Each set consisted of two test tubes of treated medium for each concentration of the substance to be tested and two tubes of the basal medium to serve as controls. One set was incubated under ordinary atmospheric air; the other set was incubated in sealed jars containing approximately 15 per cent of carbon dioxide. All test tubes were incubated at 37 C. for six days. Subcultures were made following 72 and 144 hours of incubation in order to determine whether the eventual inhibition was bactericidal or bacteriostatic in nature. Subcultures were also incubated for six days.

Recording of Results.—The visible amount of growth was graded according to the depth and density of surface growth (table 1). Observations were begun 24 hours following inoculation and were repeated every 24 hours until completion of the experiment.

RESULTS

*Sodium Azide.**—This metal-binding agent has been widely employed in enzymologic research. It is one of the few enzyme inhibitors incorporated in mediums for depressing the growth of certain bacteria. While it is generally accepted that this agent inhibits glycolysis, the exact mode and site of action are not known.[26, 32, 46, 68] Sodium azide appears to have only a limited effect on protein metabolism.[29, 66, 72] Lichstein and co-workers [40, 63] demonstrated the inhibitory effect of sodium azide on the growth of several gram-negative bacteria, including *Ps. aeruginosa* and *P. vulgaris*.

Sodium azide in this experiment was incorporated in mediums in 0.01 per cent, 0.015 per cent, 0.02 per cent, and 0.03 per cent concentrations. All three species studied were shown to have increased sensitivity to the inhibitor under 15 per cent carbon dioxide tension. *Vibrio fetus* was found to be most sensitive. Although 0.015 per cent

* Sodium azide (Practical), supplied by Eastman Organic Chemicals, Rochester, N.Y.

of the inhibitor was found to be bactericidal in atmospheric air, 0.01 per cent concentration was sufficient to kill all organisms incubated under 15 per cent carbon dioxide. None of the concentrations of sodium azide studied were bactericidal for *Ps. aeruginosa* and *P. vulgaris*. Increase of sodium azide concentration to 0.03 per cent was required in order to inhibit the growth of *Ps. aeruginosa* when incubated under 15 per cent carbon dioxide tension.

Sodium Fluoride.—This agent is another frequently studied metal-binding enzyme inhibitor. Since establishment of its effect on enolase,[44] it has been shown to inhibit several enzymes in the tri-carboxylic acid cycle.[4] Phosphogluconic acid dehydrogenase has been found not to be affected by sodium fluoride.[26]

The concentrations studied failed to cause complete inhibition of growth of *P. vulgaris* and *Ps. aeruginosa*. Slight but increasing depression of growth with increased concentrations of the inhibitor was observed in both species, *P. vulgaris* being the more sensitive.

Mercuric Chloride.—Fildes[19] demonstrated that the anti-bacterial action of mercuric chloride is due to interference with sulfhydryl (SH) groups. Mercuric chloride sensitivity of several enzymes involved in carbohydrate and protein metabolism has been reported by several workers.[45, 47, 55, 68]

Mercuric chloride was employed in this experiment in 0.001 per cent, 0.0005 per cent, 0.00025 per cent, 0.000125 per cent, and 0.0000625 per cent concentrations. A

TABLE 2—Per Cent Concentrations of Enzyme Inhibitors Required for Complete Inhibition of Growth of Vibrio fetus, Proteus vulgaris, and Pseudomonas aeruginosa

	Vibrio fetus		*Proteus vulgaris*		*Pseudomonas aeruginosa*	
Enzyme inhibitor	Atmos. CO_2 tens.	Incr. CO_2 tens.	Atmos. CO_2 tens.	Incr. CO_2 tens.	Atmos. CO_2 tens.	Incr. CO_2 tens.
Sodium azide	0.02	0.015	0.03	0.02	0.03*	0.03
Sodium fluoride	0.5	0.2	0.5*	0.5*	0.5*	0.5*
Semicarbazide	0.5**	0.5**	1.0**	1.0**	1.0**	1.0**
Hydroxylamine nitrate	0.025	0.025	0.1**	0.1**	0.1**	0.1**
Mercuric chloride	0.0000625	0.0000625	0.001**	0.001**	0.001**	0.001**
p-Chloromercuric benzoate	0.001	0.001	0.05*	0.05*	0.05*	0.05*
Copper sulfate	0.1	0.1	0.25	0.25	0.25	0.25
Sodium arsenite	0.02	0.02	0.1*	0.1*	0.1*	0.1*
Iodoacetic acid	0.05	0.05	0.15	0.1	0.15	0.1
Iodosobenzoic acid	0.1	0.1	0.1*	0.1*	0.1*	0.1*
2,4-dinitrophenol	0.015	0.015	0.2	0.2	0.2	0.2
EDTA***	0.05	0.05	0.1	0.1	0.5*	0.5*
2,6-diaminopurine	0.2	0.2	0.2*	0.2*	0.2*	0.2*
Malonic acid	0.075	0.075	0.1*	0.1*	0.1*	0.1*
Isonicotinic acid hydrazide	0.6	0.6	0.6*	0.6*	0.6*	0.6*
N-ethylmaleimide	0.05	0.05	0.1	0.1	0.15	0.15
Benzalkonium chloride†	0.0025	0.0025	0.025	0.025	0.05**	0.05**
TEADA‡	0.15	0.15	5.0*	5.0*	5.0*	5.0*
Triphenyl tetrazolium chloride	0.025	0.05	0.5	0.5	2.0*	2.0*

* The inhibitory level not determined. ** The effect observed in subcultures. *** Disodium ethylenediamine tetra-acetate. † Alkyldimethylbenzylammonium chlorides (zephiran). ‡ Triethanolamine-duodecylbenzyl sulfonate.

Concentrations of 0.2, 0.3, 0.4, and 0.5 per cent sodium fluoride were employed in this study. As in the case of sodium azide, increased carbon dioxide tension increased the potency of this inhibitor regardless of the species of bacteria studied. Although 0.2 per cent of sodium fluoride caused complete inhibition of *V. fetus* cultivated under increased carbon dioxide tension, 0.5 per cent concentration was required for complete depression of growth in ordinary atmospheric environment. Sodium fluoride exerted a bactericidal effect on *V. fetus*.

yellowish-brown precipitate was formed upon contact of the mercurial with the basal medium, thus preventing observations in the original cultures. Subcultures were made from all tubes after 72 and 144 hours of incubation to determine the effect of the exposure of the inoculums to mercuric chloride. All concentrations tested proved to be bactericidal to *V. fetus*, but unrestricted growth occurred in subcultures of *P. vulgaris* and *Ps. aeruginosa*. Carbon dioxide tension did not influence the effect of the inhibitor.

*p-Chloro-Mercuri-Benzoate.**—This organic mercurial is generally classified as the most potent of heavy-metal enzyme poisons. The reported inhibitory range includes practically all SH-enzymes.[10, 25, 42, 60, 61]

Concentrations of 0.001 per cent, 0.005 per cent, 0.01 per cent, and 0.05 per cent p-chloro-mercuri-benzoate were incorporated in the test mediums. All concentrations killed *V. fetus*. The growth of *P. vulgaris* and *Ps. aeruginosa* was not affected by the two lower concentrations. Temporary retardation of growth, lasting for 72 hours, was observed at the 0.01 per cent level of the inhibitor. The 0.05 per cent concentration caused depression of growth of both bacteria throughout the entire observation period. Influence of carbon dioxide tension was not observed.

Copper Sulfate.—Copper sulfate, another heavy-metal enzyme inhibitor, has been found to affect several steps of protein metabolism.[66, 68] Stumpf [67] observed inactivation of pyruvic acid oxidase of *P. vulgaris*, an enzyme not affected by high concentrations of sodium azide, sodium fluoride, iodobenzoate, and sodium arsenite. Copper sulfate has been reported to inactivate phosphogluconic acid dehydrogenase, an enzyme not inhibited by high concentrations of sodium fluoride and sodium azide.[26]

In this experiment, copper sulfate was incorporated in 0.025 per cent, 0.05 per cent, 0.1 per cent, and 0.15 per cent concentrations. The two lowest levels of copper sulfate produced marked retardation of growth of *V. fetus* when incubated under 15 per cent carbon dioxide tension. These levels, however, failed to depress the growth of this organism when cultivated aerobically. Appearance of growth of *V. fetus* was delayed for 96 hours in presence of 0.1 per cent of the inhibitor, regardless of carbon dioxide tension. Concentrations below 0.1 per cent did not inhibit the growth of *P. vulgaris* and *Ps. aeruginosa*. Depression of surface growth and absence of depth growth of the latter bacteria was observed at the 0.1 per cent level of copper sulfate. This effect was not influenced by differences in carbon dioxide tension. The 0.25 per cent concentration of the inhibitor was bactericidal for all three species of bacteria studied.

Sodium Arsenite.—It has been shown that this enzyme inhibitor, which affects sulfhydryl groups, acts upon several reactions involving pyruvic acid in the tricarboxylic acid cycle.[31, 65] Sodium arsenite is also known to inhibit metabolism of certain amino acids and fatty acids.[23, 30, 42, 47]

Sodium arsenite was employed in this study in 0.02 per cent, 0.04 per cent, 0.08 per cent, and 0.1 per cent concentrations. All levels of the inhibitor proved to be bactericidal for *V. fetus*. The 0.02 per cent and 0.04 per cent concentrations did not affect the growth of *P. vulgaris* and *Ps. aeruginosa*. The two higher levels of sodium arsenite caused marked depression of growth of both organisms for 72 hours, during which period only surface growth was observed. Unrestricted growth throughout the tube appeared at the 96-hour observations.

2,4-Dinitrophenol.—This widely used enzyme inhibitor is considered to act as an uncoupling agent. Its effect on several reactions of carbohydrate and protein metabolism has been reported by a great number of authors.[4, 11, 13, 20, 29, 49]

The 2,4-dinitrophenol was employed in two sets of concentrations. The set of lowest concentrations, ranging up to 0.002 per cent of the inhibitor, did not exert any adverse effect on the growth of bacteria tested. It appeared that the growth of *V. fetus* was slightly stimulated. In the second experiment, 2,4-dinitrophenol was incorporated in 0.015 per cent, 0.02 per cent, 0.15 per cent, 0.2 per cent, and 0.5 per cent concentrations. These higher levels of the inhibitor completely inhibited *V. fetus*, and proved to be bactericidal. *Proteus vulgaris* and *Ps. aeruginosa* were found to have distinct differences in sensitivity to the inhibitor. The 0.15 per cent level of the inhibitor produced bactericidal effect on *P. vulgaris*, but the growth of *Ps. aeruginosa* was not altered. Complete inhibition (bactericidal in effect) of growth of the latter bacteria was obtained with an 0.2 per cent concentration of 2,4-dinitrophenol.

*Iodosobenzoic Acid.**—Iodosobenzoic acid is an enzyme inhibitor that prevents reduction of enzymes catalysing oxidation reduction reactions.[41, 61] Krebs [35] reported that iodosobenzoic acid is a selective inhibitor of d-amino acid oxidase.

The concentrations of iodosobenzoic acid used in this study were 0.025 per cent, 0.05 per cent, 0.075 per cent, and 0.1 per cent.

* Supplied by Sigma Chemical Co., St. Louis, Mo.

* Supplied by Sigma Chemical Co., St. Louis, Mo.

The growth of *V. fetus* was not affected by the lowest concentration of the inhibitor. Depression of growth was noticeable at the 0.05 per cent and 0.075 per cent levels. The 0.1 per cent concentration was lethal to *V. fetus*. The growth of *P. vulgaris* and *Ps. aeruginosa* was not inhibited by the concentrations of iodosobenzoic acid employed.

Iodoacetic Acid.—This sulfhydryl-binding agent has been reported to block oxidation at several sites in the glycolytic and tricarboxylic acid pathways of carbohydrate metabolism.[9, 46] Iodoacetic acid, however, does not inhibit phosphogluconic acid dehydrogenase.[26] Amino acid oxidases studied have not been affected by iodoacetic acid.[14, 30, 66]

Iodoacetic acid * was incorporated in the test mediums to obtain 0.025 per cent, 0.1 per cent, 0.15 per cent, and 0.2 per cent final concentrations. All these concentrations proved to be bactericidal for *V. fetus*. *Proteus vulgaris* and *Ps. aeruginosa* were found to have an essentially similar sensitivity to the inhibitor, the effect being strongly enhanced by incubation under 15 per cent carbon dioxide tension. Thus, the 0.1 per cent level of iodoacetate caused only temporary depression of growth in aerobic environment. The same concentration proved to be lethal to both organisms cultivated under increased carbon dioxide tension. Growth of *P. vulgaris* and *Ps. aeruginosa* was completely inhibited by the two higher levels of iodoacetic acid.

Semicarbazide.—Semicarbazide is generally considered to be a carbonyl-binding enzyme inhibitor. The available reports indicate that it acts primarily upon enzyme systems involved in amino acid metabolism.[14, 30, 47, 68]

The mediums for this study were prepared to contain 0.25 per cent, 0.5 per cent, 0.75 per cent, and 1.0 per cent of semicarbazide. Addition of semicarbazide resulted in sedimentation which prevented direct observation of results. The effect of the inhibitor was, therefore, studied in subcultures made from all tubes after 72 and 144 hours of incubation. Ordinary rate of growth of *V. fetus* was observed only in the 72-hour subcultures originating from tubes containing 0.25 per cent of semicarbazide. None of the remaining subcultures of *V. fetus* grew visibly during the 144-hour observation period. All subcultures of *P. vulgaris* and *Ps. aeruginosa* were shown to have unrestricted growth.

* Supplied by Eastman Kodak Co., Rochester, N.Y.

*Hydroxylamine Nitrate.**—This carbonyl-binding enzyme inhibitor is also reported to affect predominantly reactions involved in amino acid metabolism.[14, 30, 36, 54, 55, 68]

Concentrations of 0.025 per cent, 0.05 per cent, 0.075 per cent, and 0.1 per cent of the inhibitor were tested in this experiment. *Vibrio fetus* did not grow in the presence of hydroxylamine nitrate in any concentration employed. The effect of the inhibitor was bactericidal. Only the two highest levels caused inhibition of growth of *P. vulgaris* and *Ps. aeruginosa*. This inhibition was observed during the entire observation period. Removal of these bacteria from the toxic medium into 72- and 144-hour subcultures resulted in full growth.

Disodium Ethylenediamine Tetra-Acetate.—The agent, EDTA, belongs to the class of chelating agents which react with polyvalent metals forming relatively stable metal complexes. Few reports are available in regard to the use of this compound in biochemical and bacteriologic research. Cole[16] found that occurrence of the nonsmooth type of *Brucella abortus* was prevented in presence of EDTA in the Gerhart-Wilson medium. This effect was reversed by addition of manganese. Mahler *et al.*[42] reported inhibition of aldehyde oxidase by EDTA. The activity of the enzyme was restored by molybdic trioxide.

The disodium salt of ethylenediamine tetra-acetic acid** was incorporated to obtain 0.025 per cent, 0.05 per cent, 0.1 per cent, 0.25 per cent, and 0.5 per cent final concentrations in the medium to be tested in this experiment. *Vibrio fetus* was not affected by the lowest concentration of the chelating agent. The higher concentrations proved to be bactericidal to *V. fetus*. *Proteus vulgaris* and *Ps. aeruginosa* were shown to differ markedly in sensitivity to the chelating agent. The 0.05 per cent level caused marked depression of the growth rate of *P. vulgaris*. Higher concentrations produced complete inhibition of growth of this organism. The inhibitory effect of EDTA on *P. vulgaris* was bacteriostatic in nature. *Pseudomonas aeruginosa* was found to be more resistant, since the

* Supplied by Bio Laboratories, Inc., New York, N.Y.

** Disodium Dihydrogen Versenate, produced by Versenes Inc., Farmington, Minn.

four lowest levels did not affect the growth of this organism. Its exposure to the 0.5 per cent concentration resulted in marked temporary depression of growth which lasted only two days.

Alkyldimethylbenzylammonium Chlorides.—The mixtures of these compounds represent the active ingredients of cationic, surface-active disinfectants. Baker *et al.*[5-7] found that 1:3,000 of benzalkonium chloride caused 86 per cent inhibition of growth of *P. vulgaris*. The growth of *Ps. aeruginosa* was inhibited 97 per cent. Knox *et al.*[33] reported that bactericidal concentrations of benzalkonium chloride caused inhibition of oxidation of glucose, lactic acid, pyruvate, succinate, formate, and alanine.

Concentrations of 0.0025 per cent, 0.005 per cent, 0.01 per cent, 0.025 per cent, and 0.5 per cent of detergent* were employed in this study. Precipitation resulting from addition of the detergent in the two highest concentrations interfered with direct observation. All levels of the detergent were found to be lethal to *V. fetus*. *Proteus vulgaris* was found to be resistant to the three lowest concentrations of the compound. Subcultures of the organism, originating from test mediums containing 0.025 per cent and 0.5 per cent of the detergent, did not grow, indicating that these levels were bactericidal. *Pseudomonas aeruginosa* growth was uninhibited in the presence of levels as high as 0.025 per cent. Subcultures of *Ps. aeruginosa* from test mediums containing 0.05 per cent of the detergent did not grow visibly, indicating the lethal effect of this concentration.

*Triethanolamine-Duodecylbenzyl Sulfonate.***—This anionic quaternary ammonium agent resembles a compound tested by Baker *et al.*[5-7] and found not to affect the growth of gram-negative bacteria such as *Escherichia coli*, *P. vulgaris*, and *Ps. aeruginosa*. The lower levels were observed to stimulate the respiration rate of the latter.

The 0.15 per cent, 0.3 per cent, 0.6 per cent, 1.25 per cent, 2.5 per cent, and 5.0 per cent concentrations of the detergent employed in this study proved to be bactericidal for *V. fetus*. Slight depression of growth of *P. vulgaris* and *Ps. aeruginosa* was obtained in presence of 5.0 per cent of the agent. The lower concentrations did not affect these bacteria.

*2,3,5-Triphenyl Tetrazolium Chloride.***—Huddleson and Baltzer[27] introduced the use of this compound for differentiation of bacterial species and variants. They observed inhibition of gram-positive bacteria by concentrations higher than 0.01 per cent. According to Weinberg,[71] a concentration of 0.05 per cent was required for complete inhibition of gram-positive organisms, while *P. vulgaris* and *Ps. aeruginosa* tolerated concentrations as high as 0.2 per cent and 1.0 per cent, respectively.

Concentrations of 0.025 per cent, 0.05 per cent, 0.1 per cent, 0.25 per cent, 0.5 per cent, 1.0 per cent, and 2.0 per cent of this agent were tested in the present study. All the concentrations were lethal to *V. fetus* cultures incubated in ordinary atmospheres. The 0.025 per cent level caused only temporary depression of growth of *V. fetus* in the presence of 15 per cent carbon dioxide. Slight, temporary inhibition of *P. vulgaris* was observed at the 0.25 per cent level of this tetrazolium salt. The higher concentrations proved to be bactericidal to this bacterium regardless of carbon dioxide tension. *Pseudomonas aeruginosa* was not affected by the agent in concentrations employed in this study.

*Malonic Acid.**—Since the establishment of this structural analogue as a potent inhibitor of succinic dehydrogenase,[52] it has been shown to inhibit also the utilization of pyruvate,[34] oxidation of oxalacetate,[48] and oxidation of fatty acids.[23] Phosphogluconic acid dehydrogenase has been found to be resistant to malonate.[26]

Malonic acid was employed in this experiment in 0.025 per cent, 0.05 per cent, 0.075 per cent, and 0.1 per cent concentrations. The growth of *V. fetus* was not inhibited by the 0.025 per cent and 0.05 per cent levels of malonate. The higher concentrations were found to be bactericidal for this bacterium. None of the concentrations tested affected the growth of *P. vulgaris* and *Ps. aeruginosa*.

2,6-Diaminopurine.—This agent is one of structural analogues employed in studies

* Kabon, produced by Monarch Chemicals, Inc., Minneapolis, Minn.

** Supplied by Monarch Chemicals, Inc., Minneapolis, Minn.

* Supplied by General Chemicals, Inc., Chagrin Falls, Ohio.

** Malonic acid, purified, supplied by California Foundation for Biochemistry Research, Los Angeles, Calif.

of nucleic acid metabolism. Complete inhibition of the growth of *Lactobacillus casei*, as caused by 100 μg. of the inhibitor per milliliter of medium, has been reported to be reversed by addition of adenine, but not by that of guanine or hypoxanthine.[18]

The test medium employed in this study contained 0.05 per cent, 0.1 per cent, 0.15 per cent, and 0.2 per cent of 2,6-diaminopurine. The 0.2 per cent concentration caused complete inhibition of growth of *V. fetus*, while exposure to the lower levels of the antimetabolite resulted in temporary depression of growth of this organism. The inhibitory effect on *V. fetus* was bacteriostatic. The growth of *P. vulgaris* and *Ps. aeruginosa* was not influenced by the presence of 2,6-diaminopurine in the test medium.

Isonicotinic Acid Hydrazide.—Fox[21] was first to demonstrate the high antituberculous potency of isonicotinic acid hydrazide *in vivo*. With *in vitro* studies, workers [8,58] showed that the agent is bactericidal only to actively growing bacteria. They concluded that the drug might act as an antimetabolite, as suggested by its structural relationship with nicotinamide.

The test medium employed in this study contained 0.075 per cent, 0.15 per cent, 0.3 per cent, and 0.6 per cent of isonicotinic acid hydrazide. The lowest level of the drug did not inhibit the growth of *V. fetus*. Depression of growth was observed in mediums containing 0.15 per cent and 0.3 per cent of the agent. The 0.6 per cent level caused complete inhibition of growth of *V. fetus*. This inhibition was, however, bacteriostatic in effect. *P. vulgaris* and *Ps. aeruginosa* were resistant to the concentrations of this drug tested.

*N-Ethylmaleimide.**—Marian *et al.*[43] tested a series of structurally related compounds in regard to their effect on bacterial growth. N-ethylmaleimide was found to possess the highest antibacterial activity. This activity was greatly reduced by addition of thiolacetate, glutathione, and horse serum, suggesting that the agent reacts with essential thiol groups of bacteria.

The 0.05 per cent, 0.1 per cent, 0.15 per cent, and 0.2 per cent concentrations of the agent used in this experiment proved to be bactericidal to *V. fetus*. Complete absence of growth of *P. vulgaris* was observed in test mediums containing 0.1 per cent, 0.15 per cent, and 0.2 per cent of N-ethylamaleimide. These concentrations were found to be lethal to this bacterium. *Pseudomonas aeruginosa* was the most resistant of the bacteria studied, requiring the presence of 0.15 per cent of N-ethylmaleimide for complete inhibition and a bactericidal effect.

* Supplied by Schwarz Laboratory, Inc., Mount Vernon, N.Y.

DISCUSSION AND SUMMARY

1) Nineteen enzyme inhibitors in various concentrations were compared with respect to their effect on the growth of *Vibrio fetus*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. The results are summarized (table 2).

2) All of the *V. fetus* strains employed in this study were found to be much more sensitive to the inhibitors than were the strains of *P. vulgaris* and *Ps. aeruginosa*.

3) The greater resistance of *P. vulgaris* and *Ps. aeruginosa* to the inhibitors tested eliminates these substances as potential ingredients of selective medium intended for isolation of *V. fetus* from contaminated materials.

4) *Pseudomonas aeruginosa* proved to be more resistant than *P. vulgaris* to sodium azide, disodium ethylenediamine tetraacetate, zephiran, N-ethylmaleimide, and 2,3,5-triphenyl tetrazolium chloride. This difference in sensitivity might be utilized for the isolation of *Ps. aeruginosa* from samples contaminated with *P. vulgaris*.

5) Incubation under 15 per cent carbon dioxide tension altered the response of bacteria studied to five inhibitors. Increased sensitivity to sodium azide, sodium fluoride, and iodoacetate was observed in cultures of all three bacteria incubated under increased carbon dioxide tension, suggesting that the glycolytic pathway of carbohydrate metabolism attains greater significance for the bacteria cultivated in a partially anaerobic environment. Increased carbon dioxide tension enhanced the inhibitory effect of copper sulfate on the growth of *V. fetus*. The response of *P. vulgaris* and *Ps. aeruginosa* to copper sulfate was not influenced by carbon dioxide tension. Increased carbon dioxide tension reduced the degree of inhibition of growth of *V. fetus* to 2,3,5-triphenyltetrazolium chloride. Such a protective in-

fluence was not observed in the case of *P. vulgaris* and *Ps. aeruginosa.*

References

[1] Adler, H. C.: Diagnosis of *Vibrio fetus* Carrier Bulls by Intravaginal Inoculation of Guinea Pigs. Vet. Med., 5, (1952): 454–459.

[2] Adler, H. C.: *Vibrio fetus* Diagnosen hos tyren. Nord. Veterinaermote, 7, (1954): 209–213.

[3] Adler, H. C.: Testing for *Vibrio fetus* Infection of Danish A. I. Bulls. 3rd Internat. Cong. Anim. Reprod., Cambridge, Sec. II, (1956): 5–7.

[4] Aisenberg, A. C., and Potter, V. R.: Effect of Fluoride and Dinitrophenol on Acetate Activation in Kidney and Liver Homogenates. J. Biol. Chem., 217, (1955): 737–749.

[5] Baker, Z., Harrison, R. W., and Miller, B. F.: Action of Synthetic Detergents on the Metabolism of Bacteria. J. Exptl. Med., 73, (1941): 249–269.

[6] Baker, Z., Harrison, R. W., and Miller, B. F.: The Bactericidal Action of Synthetic Detergents. J. Exptl. Med., 74, (1941): 611–620.

[7] Baker, Z., Harrison, R. W., and Miller, B. F. Inhibition of Phospholipids of the Action of Synthetic Detergents on Bacteria. J. Exptl. Med., 74, (1941): 620–637.

[8] Barclay, W. R., Ebert, R. K., and Koch-Weser.: Mode of Action of Isoniazid. Am. Rev. Tuberc., 67, (1953): 490–496.

[9] Barron, E. S. G., and Friedman, T. E.: Oxidation by Microorganisms which do not Ferment Glucose. J. Biol. Chem., 137, (1941): 596–610.

[10] Barron, E. S. G., and Singer, P. T.: Studies on Biological Oxidations. XIX. Sulfhydryl Enzymes in Carbohydrate Metabolism. J. Biol. Chem., 157, (1945): 221–240.

[11] Bernstein, D. E.: Studies on the Assimilation of Dicarboxylic Acids by *Pseudomonas saccharophilia.* Arch. Biochem., 3, (1944): 445–458.

[12] Bryner, J. H., and Frank, A. H.: Laboratory Techniques for Isolation and Propagation of *Vibrio fetus* from Cattle. Am. J. Vet. Res., 16, (Oct., 1955): 634–635.

[13] Clifton, C. E.: On the Possibility of Preventing Assimilation in Respiring Cells. Enzymologica, 4, (1937): 246–253.

[14] Cohen, P. P., and McGilvery, R. W.: Respiratory Enzymes. Burgess Publishing Co., Minneapolis, Minn. (1950): 227–251.

[15] Cohen, H. K.: Studies Relating to the Diagnosis of Vibriosis in Cattle. Ph.D. thesis, University of Wisconsin, Madison, 1955.

[16] Cole, L. J.: Suppression of Colonial Variation in *Brucella abortus* by Metal-Coupling Agents. J. Bact., 64, (1952): 847–853.

[17] Duodoroff, M.: The Oxidative Assimilation of Sugars and Related Substances by *Pseudomonas saccharophilia.* Enzymologia, 9, (1940): 59–72.

[18] Elion, G. B., and Hitchings, H. G.: Antagonists of Nucleic Acid Derivatives in Reversal Studies with 2-Aminopurine and 2, 6-Diamino Purine. J. Biol. Chem., 187, (1950): 187–211.

[19] Fildes, P.: The Mechanism of the Antibacterial Action of Mercury. Brit. J. Exptl. Path., 21, (1940): 67–73.

[20] Foster, J. W.: Microbiological Aspects of Riboflavin. I. Introduction. II. Bacterial Oxidation of Riboflavin to Lumichrome. J. Bacteriol., 47, (1944): 27–41.

[21] Fox, H. H.: The Chemical Approach to the Control of Tuberculosis. Science, 116, (1952): 129–134.

[22] Garm, O., Hoff, H., and Skjerven, O.: Kurative Behandlings forsok af Vibriose. Nord. Vet.-med., 5, (1953): 401–416.

[23] Grafflin, A. L., and Green, D. E.: Studies on the Cyclophorase System. II. The Complete Oxidation of Fatty Acids. J. Biol. Chem., 176, (1948): 95–115.

[24] Grant, C. A.: Bovine Vibriosis. Canad. J. Comp. Med. & Vet. Sci., 19, (1955): 156–164.

[25] Hellerman, L., Chinard, F. P., and Deitz, V. R.: Protein Sulfhydryl Groups and the Reversible Inactivation of the Enzyme Urease. J. Biol. Chem., 147, (1943): 443–462.

[26] Horecker, B. L., and Smyrniotis, P. A.: Phosphogluconic Acid Dehydrogenase from Yeast. J. Biol. Chem., 193, (1951): 371–381.

[27] Huddleson, I. F., and Baltzer, B.: Differentiation of Bacterial Species and Variation within Species by Means of 2,3,5-Triphenyltetrazolium Chloride in Culture Medium. Science, 112, (1950): 651–652.

[28] Hughes, D. E.: Notes on Vibriosis with Special Reference to the Isolation of *Vibrio fetus* from Semen and Preputial Fluids. Cornell Vet., 46, (1956): 249–256.

[29] Judah, J. D.: The Action of 2,4-Dinitrophenol on Oxidative Phosphorylation. Biochem. J., 49, (1951): 271–285.

[30] Kallio, R. E., and Porter, J. R.: The Metabolism of Cystine and Cysteine by *Proteus vulgaris* and *Proteus morgani.* J. Bacteriol., 60, (1950): 607–615.

[31] Kaltenbach, J. P., and Kalnitsky, B.: The Enzymatic Formation of Oxalacetate from Pyruvate and Carbon Dioxide. II. J. Biol. Chem., 192, (1951): 641–649.

[32] Kaplan, N. C.: Thermodynamics and Mechanisms of the Phosphate Bond. In "The Enzymes." The Academic Press, Inc., New York, Vol. 2, part 1 (1951): 89.

[33] Knox, W. E., Auerbach, V. A., Zaruduaya, K., and Sprites, M.: The Effects of Cationic Detergents on Bacteria and Bacterial Enzymes. J. Bact., 58, (1949): 443–452.

[34] Krebs, H. A., and Eggleston, L. V.: The Oxidation of Pyruvate in Pigeon Breast Muscle. Biochem. J., 34, (1940): 442–459.

[35] Krebs, H. A.: Oxidatior of Amino Acids. In "The Enzymes." The Academic Press Inc., New York, Vol. 2, pa t 1 (1951): 500.

[36] Kun, E.: *In Vitro* Oxidation of Glycolic Acid and Glycolaldehyde by Liver Enzymes. Fed. Proc., 11, (1952): 364.

[37] Kuzdas, C. D., and Morse, E. V.: A Selective Medium for the Isolation of *Vibrio fetus* and Related Vibrios. J. Bact., 77, (1956): 251–252.

[38] Lawson, J. R., and McKinnon, D. J.: *Vibrio fetus* Infection in Cattle. Vet. Rec., 64, (1952): 763–771.

[39] Lerche, P.: Beitrag zum Spirillen-Abortus beim Rinde. Deutsche Tierärztl. Wchschr., 30, (1927): 484–487.

[40] Lichstein, H. C., and Soule, M. K.: Studies of the Effect of Sodium Azide on Microbic Growth and Respiration. J. Bact., 47, (1944): 221–257.

[41] Lotspeich, W. D., and Peter, R. A.: The Action of Sulfhydryl Inhibitors Upon Isocitric Dehydrogenase with Special Reference to the Behavior of Some Trivalent Arsenicals. Biochem. J., 49, (1951): 704–709.

[42] Mahler, D. K., Mackler, B., and Green, D. E.: Studies on Metalloflavoproteins. III. Aldehyde Exidase, a Molybdoprotein. J. Biol. Chem., 210, (1954): 210–465.

[43] Marrian, D. K., Freedman, E., and Ward, L. J.: The Antibacterial Effects of Substances Structurally Resembling Maleimide. Biochem. J., 54, (1953): 65–68.

[44] Meyerhof, O., and Junowicz-Kocholaty, R.: The Twofold Activation of Carbohydrate Breakdown by Arsenate and the Dephosphorylation of Phosphopyruvic Acid. J. Biol. Chem., 145, (1942): 443–455.

[45] Moyed, W. S., and O'Kane, D. J.: Fractionation of the Pyruvate Oxidase of Proteus Vulgaris. J. Biol. Chem., 195, (1952): 377–381.

[46] Nord, F. F., and Weiss, R. S.: Yeast and Mold Fermentation. In "The Enzymes." Academic Press Inc., New York, Vol. 2, part 1 (1951): 787.

[47] Oginsky, L. E., and Gehrig, F. R.: The Arginine Dihydrolase Systems of *Streptococcus fecalis.* J. Biol. Chem., 198, (1952): 799–805.

[48] Pardee, A. B., and Potter, V. R.: Malonate Inhibition of Oxidation in the Krebs Tricarboxylic Acid Cycle. J. Biol. Chem., 178, (1949): 241–250.

[49] Pinsky, M. J., and Stokes, J. L.: Requirements of Formic Hydrogenlyase Adaptation in Non-Proliferating Suspensions of *Escherichia coli.* J. Bact., 64, (1952): 151–161.

[50] Plastridge, W. N., Williams, L. F., Easterbrooks, H. L., Walker, E. C., and Beccin, R. N.: Vibriosis in Cattle. Storrs (Conn.) Agric. Exper. Sta. Bull. 281, 1951.

[51] Plastridge, W. N.: Vibriosis. Adv. Vet. Sci., 2, (1955): 326–379.

[52] Quastel, J. H., and Wheatley, A. H. M.: XVI

Biological Oxidations in the Succinic Acid Series. Biochem. J., 25, (1931): 117–127.

[58] Ristic, M., and Morse, E. V.: Experimental *Vibrio fetus* Infection in Guinea Pigs. I. Bacteriological Aspects. Am. J. Vet. Res., 14, (July, 1953): 399–404.

[54] Roberts, E.: Inhibition of Bacterial Glutamic Decarboxylase. J. Biol. Chem., 198, (1952): 495–500.

[55] Roberts, H. N., and Rahn, O.: The Amount of Enzyme Inactivation at Bacteriostatic and Bacteriocidal Concentrations of Disinfectants. J. Bact., 52, (1946): 639–644.

[56] Robinson, E. M., van Rensburg, S. W. J., van Heerden, J. S., and van Drimmelen, C. C.: Isolation of *Vibrio fetus* from Bull Semen in South Africa. J. South African Vet. M. A., 27, (1956): 197–200.

[57] Ryff, J. E., and Lee, A. M.: *Vibrio fetus* in Sheep. Am. J. Vet. Res., 6, (July, 1945): 149–158.

[58] Schaefer, W. B.: The Effect of Isonicazid on Growing and Resting Tubercle Bacilli. Am. Rev. Tuberc., 69, (1954): 125-127.

[59] Singer, T. P., and Barron, E. S.: Studies on Biological Oxidations. Sulfhydryl-Enzymes in Fat and Protein Metabolism. J. Biol. Chem., 157, (1945): 241–253.

[60] Slater, E. C.: A Respiratory Catalyst Required for the Reduction of Cytochrome c by Cytochrome b. Biochem. J., (1949): 14–30.

[61] Slater, E. C.: Effect of Sulfhydryl-Combining Compounds on the Activity of the Succinic Oxidase System. Biochem. J., 45, (1949): 130–142.

[62] Smith, T.: Spirilla Associated with Disease of Fetal Membranes in Cattle. J. Exptl. Med., 28, (1918): 701–719.

[63] Snyder, M. L., and Lichstein, H. C.: Sodium Azide as an Inhibitory Substance for Gram-Negative Bacteria. J. Infect. Dis., 67, (1940): 113–115.

[64] Stedman, R. L., and Kravitz, E.: Evidence for a Common Pathway for Pyruvate and Acetate Oxidation by *Micrococcus pyogenes* var. *aureus*. Arch. Biochem. & Biophys., 59, (1955): 260–268.

[65] Steger, G.: Beobachtungen zur Vibriosis Genitalis beim Rind. Deutsche Tierärztl. Wchschr., 6, (1956): 37–41, 49–54, 61–65, 73–76.

[66] Stumpf, P. K., and Green, D. E.: 1-Amino Acid Oxidase of *Proteus vulgaris*. J. Biol. Chem., 153 (1944): 387–399.

[67] Stumpf, P. K.: Pyruvic Oxidase of *Proteus vulgaris*. J. Biol. Chem., 159, (1945): 529.

[68] Taylor, E. S., and Gale, E. F.: Studies on Bacterial Amino Acid Decarboxylases. 6. Codecarboxylase Content and Action of Inhibitors. Biochem. J., 39, (1945): 52–58.

[69] Terpstra, J. I., and Eisma, W. A.: *Vibrio Foetus* Infection in Cattle and Enzootic Infertility. Tijdschr. Diergeneesk., 76, (1951): 433–447.

[70] Terpstra, J. I.: Vibriose Bovine. XXII[ieme] Session du Domited de l'office. Office Intern. des Epizooties, Paris, (1954): 640–648.

[71] Weinberg, E. E.: Selective Inhibition of Microbial Growth by the Incorporation of Triphenyl Tetrazolium Chloride in Culture Medium. J. Bact., 65, (1953): 240–242.

[72] Zeller, E. A.: Oxidation of Amines. In "The Enzymes." The Academic Press, Inc., New York. Vol. 2, part 1 (1951): 536.

SUMMARIO IN INTERLINGUA

Le Effecto, Producite per Inhibitores de Enzyma, in *Vibrio fetus*, *Proteus vulgaris*, e *Pseudomonas aeruginosa*

1. Dece-novem inhibitores de enzyma in varie concentrationes esseva comparate in lor effecto super le crescentia de *Vibrio fetus*, *Proteus vulgaris*, e *Pseudomonas æruginosa*. Le resultatos es summarisate (Vide tabula 2).

2. Esseva trovate que omne le racias de *V. fetus* usate in iste studio esseva multo plus sensibile al effecto del inhibitores que le racias de *P. vulgaris* e de *Ps. æruginosa*.

3. Le plus grande resistentia de *P. vulgaris* e *Ps. æruginosa* contra le inhibitores testate elimina iste substantias como ingredientes possibile de medios selective destinate al isolation de *V. fetus* ab materiales contaminate.

4. *Ps. æruginosa* se provava plus resistente que *P. vulgaris* contra azido de natrium, ethylenediaminetetra-acetato dinatric, zephirano, N-ethylmaleimida, e chloruro de tetrazolium 2,3,5-triphenylic. Iste differentia in sensibilitate es possibilemente utilisabile in le isolation de *Ps. æruginosa* ab specimens contaminate con *P. vulgaris*.

5. Le incubation sub un tension de 15 pro cento de bioxydo de carbon alterava le responsa del bacterios studiate a cinque del inhibitores. Un augmento del sensibilitate pro azido de natrium, fluoruro de natrium, e iodo-acetato esseva observate in culturas de omne le tres bacterios quando illos esseva incubate sub un augmentate tension de bioxydo de carbon. Isto pare indicar que le via glycolytic del metabolismo de hydrato de carbon attinge un plus alte signification pro le bacterios quando illos es cultivate in un partialmente anaerobie ambiente. Le augmento del tension de bioxydo de carbon promoveva le effecto inhibitori de sulfato de cupro super le crescentia de *V. fetus*. Le responsa de *P. vulgaris* e de *Ps. æruginosa* a sulfato de cupro non esseva influentiate per le tension de bioxydo de carbon. Le augmento del tension de bioxydo de carbon reduceva le grado del inhibition del crescentia de *V. fetus* per chloruro de tetrazolium 2,3,5-triphenylic. Un simile effecto protectori non esseva observate in le caso de *P. vulgaris* e de *Ps. æruginosa*.

Tuberculin Reaction Size as Related to the Number of Simultaneous Tuberculin Injections

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MULTIPLE intradermal injections are often given to animals sensitized to the products of the tubercle bacillus for evaluating test products.[1,3] In addition, several sites are sometimes used when herds with tuberculin reactors in previous tests are retested. Crumb *et al.*[2] investigated differences in reactions when three different protein fractions were tested one at a time or several at a time on guinea pigs. They found that differences between single and multiple tests were small, except that reactions from one protein fraction were smaller when it was given with other substances than when it was given alone.

It had been observed at this laboratory that reactions on cattle with a low sensitivity to tuberculin appeared to be smaller when several injections of tuberculin were made than had been shown on previous occasions when only one injection had been made. Kuttler[5] made a similar observation. The following experiments were undertaken to determine if the number of simultaneous tuberculin injections in cattle would affect the size of each reaction.

MATERIALS AND METHODS

Experiment 1.—Nine cattle were each sensitized with subcutaneous injections of 50 mg. of killed *Mycobacterium tuberculosis* var. *bovis* (Bacillus Calmette-Guerin) in sterile mineral oil. In addition, each was given booster injections of the same product at intervals during the experiment to maintain adequate sensitivity. These cattle were used to determine the average reaction size that could be elicited by 4, 32, or 60 simultaneous injections of 0.2 ml. of tuberculin.* The skin thickness of each injection site was measured in millimeters before injection and 48 and 72 hours afterward with a dermic thickness gauge.[4] The increases in skin thicknesses were recorded as reaction sizes. The sensitizing injections were given to the cattle in a change-over design using two 3 by 3 Latin squares in order to measure a residual as well as direct effects of the injections. Six intradermal tests, identified as tests 1 through 6, were conducted on each animal. The first test was conducted 30 days after sensitization and the remaining five tests were made at approximately 30-day intervals.

In the first test, 3 animals were each given four intradermal injections of tuberculin, 3 were each given 32 injections, and the remaining 3 were given 60 injections. The tuberculin injections were evenly distributed over four regions of sensitivity of the animal's body,[6] excluding the caudal fold region. When only four injections were given, one was made in each of the four regions to prevent any confusion in determining reaction size and site differences. The individual animals were selected at random to decide the number of injections each would be given on the first test.

TABLE 1—Experimental Design and Testing Sequence Used to Study Simultaneous Tuberculin Injections

Test No.	No. of injections per cow		
	Cow 1	Cow 2	Cow 3
EXPERIMENT 1			
1	4	32	60
2	32	60	4
3	60	4	32
EXPERIMENT 2			
7	1	2	4
8	4	1	2
9	2	4	1

After each test, the animals were rotated so that each was given a different number of injections on each subsequent test.

At the completion of the third test, each animal had been given each series of injections (*i.e.*, 4, 32, and 60). Thirty days after the completion of the third test, the animals were selected at random and each was tested the fourth, fifth, and sixth times. Cattle were rotated between tests as in the first three tests. Examples of the sequence of testing for 3 of the cattle are shown (table 1).

Experiment 2.—In this experiment, a second group of 9 cattle was used for six tests, identified as tests 7 through 12. In these tests, the average

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* Tuberculin was supplied by the Animal Disease Eradication Division, ARS, USDA.

TABLE 2—Reaction Size Following Multiple, Simultaneous Injections of Tuberculin

Test No.	No. of injections per cow	48 hours: Range in size (mm.)	48 hours: Average size (mm.)	72 hours: Range in size (mm.)	72 hours: Average size (mm.)
EXPERIMENT 1					
1 through 6	4	1.0–21.4	7.28	1.0–19.2	6.28
	32	1.0–19.2	4.13	0.0–12.0	3.30
	60	0.0– 9.0	3.19	0.0– 6.8	2.56
EXPERIMENT 2					
7 through 12	1	2.6–23.6	10.63	2.6–18.4	9.41
	2	2.0–18.0	8.36	4.2–18.0	7.65
	3	1.2–16.8	7.48	1.4–11.8	6.68

reaction size elicited by one, two, or four simultaneous injections of intradermal tuberculin was determined. Each animal was sensitized with 50 mg. of *M. tuberculosis* var. *bovis*. The first test was conducted 30 days after sensitization, and the remaining tests were conducted at approximately 30-day intervals. In test 7, 3 cattle were each given one injection of tuberculin, 3 were each given two injections, and 3 were given four injections each. In this experiment, all injections were made in the medium sensitive skin region of the side since there was adequate room for them in that region. The method used to measure reaction size was identical to that used in experiment 1. As in experiment 1, all animals were rotated after each test. At the completion of the third test, each animal had been given each series of injections (*i.e.*, 1, 2, and 4). At this time they were again selected at random and given the three additional tests (10 through 12). Examples of the sequence of testing for 3 cattle are shown (table 1).

RESULTS AND DISCUSSION

The readings were tabulated and evaluated by an analysis of variance in accordance with a method outlined by Cochran and Cox.[7] The 48- and 72-hour readings are shown in millimeters for each treatment in each experiment (table 2). In both experiments, the average size of the reactions became progressively smaller as the number of simultaneous injections was increased. By the analysis of variance (table 3) it was seen that this decrease in size was an effect resulting from the increased number of injections and not due to chance. It was also seen that a 30-day interval between tests was adequate to prevent the residual effects of a test having an effect on a subsequent test. From the results obtained in experiment 1, it appears that careful consideration must be given to the number of intradermal injections made on an animal sensitized to products of the tubercle bacillus when evaluating new test products.

The results obtained in experiment 2 indicate that for tuberculin testing in the field only one injection of tuberculin should be made at a given time.

TABLE 3—Analyses of Variance for 48- and 72-Hour Readings (Mean Squares Only)

Source of variation	d.f.	Mean squares: 48-hour readings	Mean squares: 72-hour readings
EXPERIMENT 1			
Series (E)§	1	128.0664**	126.1028**
Squares (S:E)†	4	5.4881	2.5760
Periods (P)‡	2	9.8042	6.1806
EP	2	13.5492*	10.9670*
SP:E	8	2.7696	2.6965
Animals (A:ES)	12	10.5402*	8.5576**
Treatments (T)	2	64.3168**	51.1392**
Residual (R)	2	2.3121	1.9020
Error	20	3.2908	2.0019
EXPERIMENT 2			
Series (E)	1	539.6017**	276.0365**
Squares (S:E)	4	25.3909	22.9432
Periods (P)	2	83.3378**	61.7974**
EP	2	39.0538*	55.0838**
SP:E	8	11.7671	7.3914
Animals (A:ES)	12	10.7925	10.8336
Treatments (T)	2	52.9169**	36.5023**
Residual (R)	2	6.1188	4.5815
Error***	18	8.8011	4.7612

The average squares for injections and residual effects are adjusted for both injection and residual effects.
* Indicates significance at the 0.05 level of probability.
** Indicates significance at the 0.01 level of probability.
*** Two missing values were estimated in this experiment due to booster injections being given only to 2 animals at one time. § A set of six tests. † Scheme of rotation of tests from one period to the next. ‡ Time lapse between tests.

An experiment on cattle naturally infected with bovine tuberculosis should be conducted to definitely confirm these results. In addition, such a test should be conducted on cattle naturally infected with Johne's disease.

SUMMARY

Eighteen cattle, sensitized with *Mycobacterium tuberculosis* var. *bovis* in mineral oil, were tested with intradermal tuberculin. The number of intradermal injections given to an individual animal in a given test

varied from 1 to 60. Thirty days after each test, the animals were rotated so that each was given a different number of injections on the subsequent test. For example, an animal given four injections in one test might be given 60 in the next.

It was found that as the number of simultaneous tuberculin injections increased, the average size reaction that each would elicit decreased.

References

[1] Baisden, L. A., Larsen, A. B., Johnson, H. W., and Vardaman, T. H.: The Influence of Aging on the Potency of Mammalian Intradermal Tuberculins. Am. J. Vet. Res., 13, (Jan., 1952): 62.66.

[2] Crumb, C., Meier, P., and Seibert, F. B.: Effects of Simultaneous Skin Tests on Size of Tuberculin Reactions. Am. Rev. Tuberc., 65, (1952): 201–205.

[3] Jones, M. A., Larsen, A. B., Vardaman, T. H., and Baisden, L. A.: Studies on the Fractionation of Tuberculin and Johnin. II. Extraction of Tuberculin Proteins with Ph nol. Am. Rev. Tuberc., 68, (1953): 439–443.

[4] Kummer, F. A., and Johnson, H. W.: Studies on Johnin. IV. Dermal Thickness Gauge. Am. J. Vet. Res., 5, (April, 1944): 189–192.

[5] Kuttler, K. L.; University of Nevada, Reno. Personal communication, (1955).

[6] Larsen, A. B., Groth, A. H., and Johnson, H. W.: Allergic Response to Johnin and Tuberculin of Various Skin Regions of Cattle. Am. J. Vet. Res., 11, (July, 1950): 301–303.

[7] Cochran, W. G., and Cox, G. M.: Experimental Designs. 2nd ed., John Wiley & Sons, Inc., New York, N.Y.

SUMMARIO IN INTERLINGUA

Magnitude del Reaction a Tuberculina in Su Relation al Numero del Simultanee Injectiones de Tuberculina

Dece-octo pecias de bestial, sensibilisate con *Mycobacterium tuberculosis* var. *bovis* in oleo mineral, esseva testate con tuberculina intradermal. Le numero del injectiones intradermal administrate al animales individual in le tests individual variava inter 1 e 60. Trenta dies post un test, le animales esseva rotate de maniera que omne animal individual recipeva un altere numero de injectiones in le subsequente test. Per exemplo, un animal que recipeva quatro injectiones in un del tests recipeva forsan 60 in le proxime.

Esseva trovate que como le numero del injectiones simultanee de tuberculina cresceva, le magnitude medie del reaction evocate per cata un se monstrava reducite.

Use of Chicks for Detecting Western Equine and St. Louis Encephalitis Viruses in Mosquitoes

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MICE of the neutrotropic, virus-susceptible, Webster strain (Swiss-W) are the test animals most commonly used for isolating western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses from biological specimens.[11] Unfortunately, mice of the Swiss-W strain are not always available from commercial sources. Under such circumstances, the research worker must either raise his own mice or use other test animals. In this study, young chicks were tested and found to be highly sensitive indicators of WEE and SLE viruses in mosquitoes.

The use of chicks instead of mice for encephalitis studies was first suggested by Chamberlain *et al.*,[6] who reported that the l.d.$_{50}$ titers of WEE and eastern equine encephalomyelitis (EEE) viruses were 10 to 1,000 times greater in 12-hour-old chicks inoculated subcutaneously than in 3- to 4-week-old Swiss mice inoculated intracerebrally. Chicks at this age usually died within 72 hours after being inoculated with either agent. Chicks inoculated more than 12 hours after hatching were more resistant to disease and, at 24 hours or older, were usually refractory. Quite different results were obtained in similar tests in which SLE virus was used. Regardless of age, chicks infected with SLE rarely had signs of disease. Chicks, therefore, were considered by these workers to be unsuitable for tests in which SLE virus was used. Their findings suggested that 12-hour-old chicks might be more sensitive than Swiss-W mice for isolating WEE virus from mosquitoes, and that chicks of this age could be substituted for mice in WEE virus-neutralization tests. A number of studies [1-3, 12, 13, 17] in which chicks were successfully used for these purposes have been reported by personnel of our research unit. However, mice were believed necessary for all studies related to SLE virus, which is also enzootic in Weld County, Colorado, our major study area.[9]

It had been known since 1941 that chickens could be infected with SLE virus and that they would subsequently develop specific neutralizing antibodies.[10] These findings suggested that young chicks might also be used for detecting SLE virus in mosquitoes if, instead of disease, the development of antibodies in test chicks were used as evidence of their infection with the virus. Obviously, to concur with our original objective, it was necessary to use a serologic technique other than the virus neutralization test in mice. For the latter purpose, the hemagglutination-inhibition (HI) test was evaluated and subsequently adopted. Described herein are studies bearing on these problems and, subsequently, on the use of chicks for demonstrating viruses in wild mosquitoes.

MATERIALS AND METHODS

Stock Viruses.—The Rockefeller strain of WEE virus and the Webster strain of SLE virus (both furnished by Dr. W. McD. Hammon) were used as prototypic viruses for neutralization tests in mice and for preparing hyperimmune serums in rabbits. Both agents were obtained as mouse brain suspensions and had three passages in 3- to 4-week-old mice in this laboratory. Two viruses ($L_{a}34_{a}$ and Texas MB) were used to infect chicks. Both had been obtained from the Virus and Rickettsia Laboratory of the Communicable Disease Center, Montgomery, Ala. The $L_{a}34_{a}$ strain (WEE) had been originally recovered from mosquitoes and had four animal passages: two in mice, one in chicken embryos, and one in 12-hour-old chicks. The Texas MB strain (SLE) had also been isolated from mosquitoes and, when used, had six passages in mice.

Test Animals.—The chicks usually employed were the 1956 progeny of a flock of crossbred, meat-type, chickens hatched in December, 1955. Chickens hatched prior to the winter of 1955 were excluded as parent stock because of their possible exposure to WEE or SLE virus during a previous epizootic season. This precaution was taken because it had been shown [15, 16] that WEE and SLE antibodies are transferred from adult birds to their progeny by transovarian passage.

Swiss-W mice were obtained from a local supplier and were usually 3 to 4 weeks old when used.

Virus Isolation from Mosquitoes.—Unengorged *Culex tarsalis* (Coquillett) mosquitoes were captured during daylight hours in Weld County, Colorado, and transported to the laboratory in chilled ice chests. Within 18 hours, the mosquitoes were anesthetized lightly with ether, identified as to species, transferred in pools of 10 to 50 to glass vials which were then hermetically sealed, and stored in a solid CO_2 chest until tested for the presence of virus. The methods used for capturing mosquitoes and the areas from which

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collections were made will be described in a separate publication.[9]

The mosquitoes of each pool were ground in a chilled mortar, using 3 ml. of diluent consisting of 33⅓ per cent normal, heat-inactivated chicken or rabbit serum in 0.9 per cent NaCl solution buffered at pH 7.4 to 7.6 and containing a final concentration of 600 to 700 units of penicillin and 600 to 700 *gamma* of streptomycin per milliliter. The resulting crude suspensions were spun at 2,000 r.p.m. for 10 minutes in an International Model PR-2 refrigerated centrifuge. The supernatant fluid from each pool was transferred to two or three 1.0-ml. ampules, hermetically sealed, shell frozen in a CO_2-alcohol bath, and stored in a solid CO_2 chest. Subsequently, the mosquito suspensions were thawed by immersion in water at 37 C. and tested for virus both in 12-hour-old chicks and 3- to 4-week-old mice. Each suspension was inoculated into 4 chicks at the rate of 0.03 ml. per chick. Each group of 4 chicks was then held for observation in a separate cardboard box. Brains for subinoculation were obtained from all moribund or dead chicks found during a 72-hour holding period. If any of the 4 chicks given the same mosquito suspension were alive at the end of the initial holding period, they were held (a maximum of 2) in a common rearing cage with other test chicks for approximately four weeks and then tested for the development of SLE antibodies by HI or serum neutralization techniques, or both. A numbered leg band was attached to each chick held for this purpose. It was therefore possible to refer the results of serologic tests to the mosquito pool with which each chick had been inoculated. When 2 chicks survived exposure to a single mosquito suspension, their serums were pooled for serologic testing. In comparative tests, a 0.03-ml. quantity of each mosquito suspension was inoculated intracerebrally into each of 6 mice, 3 to 4 weeks old. Brains for subpassage were harvested from all mice having signs of disease or found dead during a 14-day holding period.

Identification of Mosquito Isolates.—Two methods were used for identifying each strain of WEE or SLE virus recovered from mosquitoes. The WEE isolates were identified by serum-neutralization tests in 6-hour-old chicks. The identity of each WEE isolate was further confirmed by demonstrating specific HI antibodies in the serums of chicks inoculated at 1 to 2 weeks of age with first-passage virus in chick brain suspension. The SLE virus was detected and tentatively identified by demonstrating HI antibodies in the serums of chicks inoculated 30 days previously with the original mosquito suspensions. Serum-neutralization tests in mice were used to identify all SLE isolates obtained through inoculation of mosquito suspensions into mice.

Methods Used for Infectivity and Neutralization Tests.—Serial tenfold virus dilutions were prepared in 10 per cent normal, heat-inactivated chicken or horse serum in physiologic saline solution. A separate pipette was used for each transfer. Infectivity titrations were conducted by preparing virus dilutions containing an estimated 100, 10, 1.0, 0.1, or 0.01 $l.d._{50}$ and immediately inoculating 2 chicks per dilution or 5 mice per dilution. If chicks were used, a minimum infective dose (m.i.d.) was estimated as the highest virus dilution eliciting HI antibodies in a pooled serum specimen from the 2 chicks into which it had been inoculated. When mice were used, an $l.d._{50}$ value was computed by the method of Reed and Muench.[14] Whenever the infectivity of a virus was determined concomitantly in chicks and mice, aliquots of each virus dilution, beginning with the highest, were inoculated into test animals of both species before proceeding to the next lower dilution. In neutralization tests used for identifying viruses or for detecting serum antibodies, constant amounts of serum were mixed with serial tenfold dilutions of virus. After incubation of the mixtures at 37 C. for two hours in a water bath, a 0.03-ml. aliquot of each dilution was inoculated into each of 4 12-hour-old chicks via the subcutaneous route or into each of 5 weanling mice via the intracerebral route. Neutralization indexes (NI) were computed by standard methods.[13]

Hemagglutination-Inhibition Techniques.—The method used for conducting HA tests was a modification of the procedure described by Casals and Brown[4,5] and Clark and Casals.[6] The following minor variations in Casals' method were employed: (1) WEE antigen was prepared from plasma obtained from moribund chicks that had been infected with the $L_{26}34_a$ strain 12 hours or less after hatching; (2) serums were treated for removal of nonspecific inhibitors by acetone precipitation as suggested by Chanock and Sabin[7]; (3) serum-antigen mixtures were incubated overnight in an ordinary mechanical refrigerator prior to adding chick erythrocytes; and (4) tubes containing serum, antigen, and erythrocytes were placed in a bacteriologic incubator at 37 C. for 1½ hours, after which the test results were observed.

Experimental Procedure.—Preliminary studies were made to determine the susceptibility of 12-hour-old and 1- to 2-week-old chicks to WEE and SLE viruses and to evaluate the applicability of the HI test as a method for detecting antibodies in their serums. Recently isolated strains of WEE and SLE viruses ($L_{26}34_a$ and Texas MB strains, respectively) were titrated in chicks and, concurrently, in mice to measure the infectivity of each virus for the two species. Chicks were tested to determine the time interval following inoculation that HI antibodies for WEE and SLE were demonstrable.

In studies on the specificity of the HI reaction, hemagglutinins (HA) prepared from WEE or SLE virus were tested against serums obtained from chicks previously inoculated either with the heterologous agent or with normal mouse brain. The HI tests against WEE and SLE antigens were performed on serums obtained from the survivors of a group of chicks held in common rearing boxes after some of them had been inoculated with SLE virus, some with WEE virus, and the remainder with

XUM

normal mouse brain. The last mentioned tests were intended to determine if either agent might be transferred from chick to chick by contact during a prolonged holding period.

Subsequent to these tests, 12-hour-old chicks were used in attempts to demonstrate WEE and SLE viruses in mosquitoes collected in Weld County, Colorado, during the summer of 1956.[9] For this purpose, suspensions were prepared from 219 mosquito pools consisting of 7,265 *Culex tarsalis* (Coquillet). As indicated previously, a portion of each suspension was also inoculated into mice.

RESULTS

Comparative infectivity tests, using the $L_2 34_a$ strain of WEE virus and the Texas MB strain of SLE virus in mice and chicks, suggested that the latter hosts were at least as sensitive as the former to infection with these agents. The mouse l.d.$_{50}$ titer of the $L_2 34_a$ strain of WEE virus was $10^{-7.4}$; the m.i.d. in 1- to 2-week-old chicks was $10^{-7.7}$. In numerous tests, the l.d.$_{50}$ titer of this strain in 12-hour-old chicks was $10^{-9.0}$ or greater. In mice, the l.d.$_{50}$ titer of the Texas MB strain of SLE virus was $10^{-7.5}$; the m.i.d. in 12-hour-old chicks was $10^{-8.0}$. The 1- to 2-week-old chicks developed HI antibodies against WEE virus by postinoculation day 19. The HI antibodies against SLE virus developed somewhat more slowly in 12-hour-old chicks but high titers were always demonstrable on the 30th day postinoculation. In no instance were WEE or SLE HI antibodies detected in chicks that had been inoculated with the heterologous agent or with normal mouse brain. None of 11 chicks developed HI antibodies after being held 30 days as normal controls in rearing boxes with other chicks that had been infected with either WEE or SLE virus.

TABLE 1—Mortality Ratios in Chicks and Mice Inoculated with Original Suspensions of Culex tarsalis (Coquillet) Found Naturally Infected with Encephalitis Viruses

Mosquito pool (lab. No.)	Mortality ratio*		Virus
	Chicks	Mice	
6V486	0/4	4/6	SLE
6V516	0/4	6/6	SLE
6V536	0/4	5/6	SLE
6V539	0/4	5/6	SLE
6V551	0/4	5/6	SLE
6V552	0/4	6/6	SLE
6V554	0/4	6/6	SLE
6V550**	3/4	5/6	SLE & WEE
6V567	4/4	2/6	WEE

SLE = St. Louis encephalitis virus; WEE = western equine encephalitis virus.

* Deaths are shown in numerator; number of test animals inoculated in denominator.

** SLE and WEE viruses recovered in mice; WEE virus recovered in chicks.

TABLE 2—Antibody Reactions Elicited in Chicks Given Viral Agents Found in Culex tarsalis (Coquillet) of Weld County, Colorado, in 1956

Viral isolates	Passage*	Serum antibody**			
		Hemagglutination inhibition titer		Neutralization index	
		SLE	WEE	SLE	WEE
ST. LOUIS ENCEPHALITIS					
6V486	Original	1/320	0	220	N.T.
6V516	Original	1/320	0	65	N.T.
6V536	Original	1/1280	0	42	N.T.
6V539	Original	1/1280	0	65	N.T.
6V550	M_2	1/640	1/160	500	313
6V551	Original	1/320	0	37	N.T.
6V552	Original	1/320	0	147	N.T.
6V554	Original	1/320	0	75	N.T.
WESTERN EQUINE ENCEPHALITIS					
6V550	C_1	0	1/320	N.T.	4,670
Idem	M_2	1/640	1/160	500	313
6V567	C_1	0	1/320	N.T.	4,670

SLE = St. Louis encephalitis. WEE = western equine encephalitis. N.T. = not tested. 0 = failure of 1/10 serum dilution to inhibit hemagglutination.

* Original = original mosquito suspension; M_2 = second mouse passage; C_1 = first chick passage.

** Antibody tests were performed against prototypic SLE or WEE viral antigen. Serum neutralization tests using serums obtained from chicks inoculated with 6V550 (WEE) or 6V567 were performed in 12-hour-old chicks; the remaining were in 3- to 4-week-old mice.

Viral isolates were obtained from nine of the 219 mosquito pools tested (tables 1–3). One pool (6V550) contained both WEE and SLE viruses, seven pools SLE virus only, and one pool WEE virus only. None of the seven mosquito suspensions yielding only SLE virus caused disease in chicks; however, each killed at least 4 of 6 mice inoculated. The suspension containing only WEE virus killed all of 4 chicks and 2 of 6 mice. Each isolate elicited HI and SN antibodies following inoculation into chicks (table 2) and each was neutralized to a significant degree by homologous serum (table 3). Hemagglutination-inhibition antibodies against SLE antigen were demonstrated only in those chicks given mosquito suspensions also shown to contain SLE virus mouse inoculation.

The isolations of SLE and WEE viruses from a single mosquito suspension (6V550) are believed unusual enough to justify further description. Since the original specimen was lethal to 3 of the 4 chicks inoculated, it was initially suspected that WEE virus had been isolated. This suspicion was confirmed by results of the serologic tests used to identify the agent contained in a first-passage chick brain suspension (6V-550C_1). However, in neutralization tests using a second-passage mouse brain sus-

TABLE 3—Neutralization by Hyperimmune Serum of Viruses Isolated in Mice Following Inoculation with Culex tarsalis (Coquillet) of Weld County, Colorado, 1956

Virus	Passage*	Neutralization index**	
		SLE immune serum	WEE immune serum
ST. LOUIS ENCEPHALITIS ISOLATES			
6V486	M_2	2,950	N.T.
6V516	M_2	2,750	N.T.
6V536	M_2	2,100	N.T.
6V539	M_2	2,150	N.T.
6V550	M_2	1,290	11
Idem	M_3	11,500	5
6V551	M_2	2,130	N.T.
6V552	M_2	21,300	N.T.
6V554	M_2	5,000	N.T.
WESTERN EQUINE ENCEPHALITIS ISOLATES			
6V550	C_1	N.T.	10,000
6V567	C_1	N.T.	320

SLE = St. Louis encephalitis; WEE = Western equine encephalitis. N.T. = not tested.

* M_2 and M_3 = second and third mouse passages, respectively; C_1 = first chick passage.

** Hyperimmune serums were prepared in rabbits given standard laboratory strain of SLE or WEE virus. Twelve-hour-old chicks were used in tests employing WEE viruses, 3- to 4-week-old mice in tests employing SLE viruses.

pension (6V550M_2), SLE immune serum protected mice against 1,290 l.d.$_{50}$, and WEE immune serum protected against only 11 l.d.$_{50}$. These unexpected results motivated additional studies intended to evaluate their validity.

The WEE virus was again shown to be present in the original mosquito suspension by isolation attempts in chicks. Unfortunately, an insufficient quantity was not available for diplicate inoculations into mice. Therefore, we were unable to obtain unequivocal evidence that SLE virus was contained in the original mosquito suspension. However, strong evidence was obtained that both viruses were present in the first-passage mouse brain suspension. For this study, brains from mice found moribund following inoculation with the M_1 virus pool were allotted to two groups. Half of the brains were prepared as a 20 per cent suspension in 50 per cent WEE-immune rabbit serum in physiologic saline solution, and the remaining brains were prepared as a 20 per cent suspension in 50 per cent SLE-immune rabbit serum in physiologic saline solution. Each of the virus pools prepared by these methods was diluted in tenfold steps, and each resulting dilution was incubated at 37 C. for two hours with equal quantities of immune serum corresponding to that with which the virus pool had been prepared. The serum-virus mixtures containing WEE immune serum were inoculated into mice; those containing SLE immune serum were inoculated into chicks. Brain suspensions for virus identification were prepared from chicks and mice given a "limiting" dilution of each virus preparation. The WEE virus was identified in the resulting chick brain suspension, SLE virus in the mouse brain suspension.

Using comparable techniques, only WEE virus could be detected in the first-passage chick brain suspension.

DISCUSSION

In this study, 12-hour-old chicks were used successfully for detecting WEE and SLE viruses in mosquitoes. A small number of mice was required for the preparation of SLE hemagglutinins and for reinoculation with SLE-positive mosquitoes, thereby allowing the recovery of SLE viruses for further study.

A laboratory using chicks for isolation of WEE and SLE viruses should save substantially in cost of laboratory animals, since fewer animals are required to test each specimen and the cost per chick is usually less than half that of the cost per mouse.

Additional advantages are: (1) 12-hour-chicks are more sensitive than mice as indicators of WEE virus; (2) chicks almost always have signs of disease within 48 hours following inoculation with WEE virus, while mice may not have signs of disease until ten days or more have elapsed; (3) compared to mice, chicks infrequently die from nonspecific causes; (4) chicks are hatched in practically all areas of the United States, while Swiss-W mice cannot be obtained locally in many sections of the country.

The disadvantages are: (1) If more than one group B agent were enzootic in a study area, the HI tests, as used in this study, would not distinguish SLE antibodies from antibodies against other viruses of the group; (2) detection of SLE virus in chicks necessitates a 30-day interval between inoculation of the specimen and the removal of chick serum for HI tests, while in 3- to 4-week-old mice, only two to three weeks are required to adequately identify many isolated strains of SLE virus; (3) chicks obtained from flocks living in an enzootic area must always be suspected of circulating maternal antibodies against WEE or SLE viruses, or both. In the present study, this

problem was circumvented, at least in the early part of the mosquito season, by using a breeder flock that had been hatched during the immediately preceding inter-epizootic period. Although it is likely that some of the breeder chickens used for this study became infected and were circulating WEE or SLE antibodies, or both, during the latter part of the summer, no evidence was found that virus detection tests in chicks became correspondingly less sensitive.

SUMMARY

1) Newly hatched chickens were shown to be as sensitive as weanling mice to infection with western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses. Chicks infected with WEE virus within 12 hours after hatching developed typical signs of disease followed by death. Inapparent infections usually resulted when older chicks were given WEE virus or when chicks of any age were inoculated with SLE virus.

2) Inapparent infections with WEE or SLE viruses, or both, were detected in chicks by demonstrating the presence of hemagglutination-inhibition (HI) antibodies in their serums. These antibodies were not detected in chicks inoculated with normal mouse brain or in chicks held as normal controls in common rearing cages with chicks infected with WEE or SLE virus.

3) Twelve-hour-old chicks were used to test for the presence of encephalitis viruses in 219 mosquito pools containing 7,265 *Culex tarsalis* (Coquillet) in Weld County, Colorado, in 1956. For comparative purposes, duplicate portions of each mosquito preparation were also inoculated into 3- to 4-week-old mice. In both testing procedures, seven mosquito pools were found to contain SLE virus; one contained WEE virus. A single additional pool yielded both SLE and WEE virus.

4) The findings were discussed in regard to possible advantages and disadvantages of using chicks as test animals for detecting WEE and SLE viruses in mosquitoes.

References

[1] Bernet, Carl P., LaVeck, Gerald D., and Winn, John F.: Absence of Neutralizing Antibodies Against Western Equine Encephalomyelitis in Sera of Children with Epilepsy. J. Immunol., 75, (1955): 330–331.

[2] Blackmore, J. S., and Winn, J. R.: *Aedes nigromaculis* (Ludlow), Mosquito Naturally Infected with Western Equine Encephalomyelitis Virus. Proc. Soc. Exptl. Biol. & Med., 87, (1954): 328–329.

[3] Blackmore, J. S., and Winn, J. F.: A Winter Isolation of Western Equine Encephalitis Virus from Hibernating *Culex tarsalis* (Coquillett). Proc. Soc. Exptl. Biol. & Med., 91, (1956): 146–148.

[4] Casals, J., and Brown, L. V.: Hemagglutination with Certain Arthropod-borne Viruses. Proc. Soc. Exptl. Biol. & Med., 83, (1953): 170–173.

[5] Casals, J., and Brown, L. V.: Hemagglutination with Arthropod-borne Viruses. J. Exptl. Med., 99, (1954): 429–449.

[6] Chamberlain, Roy W., Sikes, R. K., and Kissling, R. E.: Use of Chicks in Eastern and Western Equine Encephalitis Studies. J. Immunol., 73, (1954): 106–114.

[7] Channock, R. M., and Sabin, A. B.: The Hemagglutination of St. Louis Encephalitis Virus. III. Properties of Normal Inhibition for Diagnosis of Infection. J. Immunol., 73, (1953): 337–351.

[8] Clark, Delphine H., and Casals, Jordi: Improved Methods for Hemagglutination Studies with Arthropod-borne Viruses. Proc. Soc. Exptl. Biol. & Med., 88, (1955): 96–99.

[9] Encephalitis Investigations Section, Communicable Disease Center, U.S.P.H.S., Greeley, Colo.: Unpublished data.

[10] Hammon, W. McD., Gray, J., Evans, C., and Izumi, E. M.: Western Equine and St. Louis Encephalitis Antibodies in the Sera of Mammals and Birds from an Endemic Area. Science, 94, (1941): 305.

[11] Hammon, W. McD.: Encephatlitis (Arthropod-borne Virus Encephalitides and Lymphocytic Choriomeningitis). In "Diagnostic Procedures for Virus and Rickettsial Diseases." 2nd ed. A.P.H.A. (1956): 169–206.

[12] Kaplan, William, Winn, John F., and Palmer, Dan F.: Susceptibility of the Pigeon (*Columbia livia*) to Infection with Western Equine Encephalomyelitis Virus. J. Immunol., 75, (1955): 225–226.

[13] LaVeck, Gerald D., Winn, John F., and Welch, Sarah F.: Inapparent Infection with Western Equine Encephalitis Virus: Epidemiologic Observations. Am. J. Publ. Health, 45, (1955): 1409–1416.

[14] Reed, L. J., and Muench, H.: Simple Method of Estimating Fifty Percent End Points. Am. J. Hyg., 27, (1938): 493–497.

[15] Reeves, W. C., Sturgeon, J. M., French, E. M., and Brookman, B.: Transovarian Transmission of Neutralizing Substances to Western Equine and St. Louis Encephalitis Viruses by Avian Hosts. J. Infect. Dis., 95, (1954): 168–178.

[16] Sooter, Clarence A., Schaeffer, Morris, Gorrie, Rachael, and Cockburn, T. Aidan: Transovarian Passage of Antibodies Following Naturally Acquired Encephalitis Infection in Birds. J. Infect. Dis., 95, (1954): 165–167.

[17] Winn, John F., Palmer, Dan F., and Kaplan, William: Development and Persistence of Western Equine Encephalitis Virus Antibodies in Experimentally Infected Pigeons. Cornell Vet., 47, (1957): 337–343.

SUMMARIO IN INTERLINGUA

Le Uso de Gallinas in le Detection de Virus de Encephalitis Equin Occidental e de Virus de Encephalitis St. Louis in Mosquitos

1. Esseva demonstrate que novemente covate gallinettas es tanto sensibile como muses de etate dislactabile pro infection per virus de encephalitis equin occidental (EEO) e de encephalitis St. Louis (ESL). Gallinettas inficite con virus de EEO 12 horas post lor covage disveloppava signos typic del morbo, sequite per morte. Infectiones non-apparente resultava usualmente quando le virus esseva administrate a gallinettas plus matur o quando gallinettas de non importa qual etate recipeva virus de ESL.

2. Infectiones non-apparente per virus de EEO o per virus de ESL o per ambes esseva detegite in le gallinettas per le demonstration del presentia de anticorpore inhibiente le hemagglutination in lor seros. Iste anticorpore esseva detegite in gallinettas inoculate con normal cerebro de muses e etiam in gallinettas mantenite como normal aves de controlo in cagias commun con gallinettas inficite per virus de EEO o virus de ESL.

3. Gallinettas de 12 horas de etate esseva usate in tests pro le presentia de virus de encephalitis in 219 populationes de mosquitos, continente un total de 7.265 individuos de *Culex tarsalis* (Coquillet) in le contato de Weld in Colorado in 1956. Pro objectivos de comparation, equal portiones de omne le preparatos de mosquito esseva etiam inoculate in muses de etates de inter 3 e 4 septimanas. In ambe methodos, le presentia de virus de ESL esseva constatate pro septe populationes de mosquitos. Un del populationes contineva virus de EEO. Un sol population additional contineva ambe le typos de virus.

4. Le constatationes es discutite ab le puncto de vista del avantages e disavantages del uso de gallinettas como animales de testage pro le presentia de virus de EEO e de virus de ESL in mosquitos.

Stable, Standardized, Sensitized Chicken Erythrocytes for the Polyvalent Salmonella Indirect Hemagglutination Test

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THE INDIRECT hemagglutination test developed by Keogh *et al.*[1,2] has been applied to many diseases,[4] especially those caused by the Enterobacteriaceae. The main advantages of the indirect hemagglutination system over the agglutination test are: (1) antibodies are detected earlier, rise to a high titer and persist longer, (2) polyvalent systems are practical, and (3) therapeutic agents apparently do not suppress hemagglutinins to the same extent as agglutinins.[6-8] Most studies on the indirect hemagglutination test have dealt with its application to different disease systems. Although some work has been done on the purification of the polysaccharide antigens for erythrocyte sensitization, little attention has been paid to the selection of erythrocyte donor animals and antigen strains, and stabilization of the system.

The purpose of this report is to present data which indicated that (1) diseases of donors of the erythrocytes can affect the test, but that such animals can be easily detected; (2) various Salmonella strains vary in their ability to give specific and nonspecific reactions, and suitable strains can be easily selected; and (3) sensitized erythrocyte preparations can be stabilized by simple formalin treatment to extend refrigerator life from four days to at least six months.

MATERIALS AND METHODS

Procedures for the preparation of washed erythrocytes, erythrocyte-sensitizing substances and sensitized erythrocytes and reading of the test were identical with those previously described.[5,6] The blood volumes and 45-degree sedimentation rates were obtained by standard procedures. Most of the Salmonella strains used were isolated from chicks and poults. The group-specific antiserums were obtained from Lederle Laboratories, Pearl River, N.Y. Procedures developed during the course of this study are described in the section dealing with experimental procedures.

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EXPERIMENTAL PROCEDURES AND RESULTS

Detection of Donor Birds Whose Erythrocytes Were Abnormal.—Chicken erythrocyte suspensions prepared for the indirect hemagglutination test normally sediment within an hour to form a compact "button" at the bottom of the test tube. The addition of specific antibody to the test system causes the hemagglutination of these antigen-sensitized erythrocytes which prevents "button" formation. During the course of experimental testing of chicken, turkey, and duck flocks for salmonellosis, the test as originally described [6] appeared reliable and gave uniform results for two years. When the strains of chickens obtained from the poultry plant were changed, erythrocytes from certain birds failed to sediment, thereby giving false positive reactions. Older chickens (8 to 10 months) which gave false reactions were found at necropsy to have lesions of the avian leukosis complex. However, younger chickens (as young as 6 weeks of age) which gave similar false reactions were free of lesions. Maintenance of young donor birds until 6 months of age apparently permitted the development of the lesions of the avian leukosis complex in the individuals whose erythrocytes were abnormal.

In an attempt to find a simple, rapid method for selecting donor birds whose erythrocytes were normal, standard blood volume and sedimentation rates were determined. These tests indicated some abnormalities among the older birds, but there was no direct correlation with serologic results, especially in the younger birds. The sedimentation rate of washed erythrocyte suspensions was the only reliable criterion for the selection of suitable erythrocytes. This value is the time required in minutes for the complete sedimentation of a 0.17 per cent cell suspension to form a compact "button." A 0.25-ml. aliquot of triple-washed 0.5 per cent erythrocytes was

TABLE 1—Criteria for the Selection of Erythrocytes for the Salmonella Indirect Hemagglutination Test. The Test System Used Salmonella typhimurium Antigen and Antiserums

Bird No.	Sed. rate, whole blood	Packed Cell volume (%)		Sed. rate, washed cells* (min.)		Hemagglutination titers				Suitability for IHA test
						Positive serums		Negative serums		
		r.b.c.	w.b.c.	Non-sens.	T.M.-sens.	1	2	3	4	
1	19.0	27.5	0.5	39	46	1,280	1,280	.....	20	
2	16.0	30.5	1.0	31	37	1,280	1,280	.....	20	
3	14.0	30.0	3.0	44	40	1,280	1,280	.....	40	
4	17.5	26.0	1.0	44	43	2,560	2,560	.....	40	
5	37.0	26.0	0.25	28	38	1,280	1,280	.....	20	
6	25.5	29.0	0.5	49	30	2,560	2,560	.....	20	Excellent
7	9.5	32.5	1.0	30	32	1,280	1,280	.....	40	
8	11.0	32.0	1.0	34	37	2,560	2,560	.....	40	
9	26.5	26.5	1.0	32	42	1,280	2,560	.....	40	
10	19.0	26.0	1.0	40	42	1,280	2,560	.....	40	
11	16.0	28.0	0.75	40	40	1,280	2,560	.....	40	
Mean	19.2	28.5	1.0	37.3	39	1,700	1,700	.....	32	
12	14.0	28.5	0.5	39	>90	1,280	2,560	.....	40	Fair
13	11.0	30.0	1.0	>90	43	10,240	2,560	.....	20	Poor
14	15.5	24.5	0.5	37	46	1,280	2,560	.....	>160	
15	13.0	28.0	0.5	>90	>90	>20,480	>20,480	.....	40	
16	16.0	24.5	0.5	>90	>90	>20,480	>20,480	.....	40	
17	24.5	24.0	0.8	>90	49	>20,480	2,560	.....	40	Unfit
18	20.5	24.5	0.8	>90	42	10,240	>20,480	.....	20	
19	16.0	26.5	0.8	>90	42	>20,480	5,120	.....	40	
Mean	18.0	25.5	0.7	>90	>62	>18,330	>10,240	.....	36	

* Time required for the complete sedimentation of triple-washed erythrocytes in a 0.17 per cent suspension.

added to 0.5 ml. of buffer in 10-mm. diameter tubes. Serums used to test the sedimentation rates of sensitized erythrocytes included two samples from naturally infected birds as well as two from uninfected birds which varied in their nonspecific titers.

Results of the determinations of sedimentation rates, blood volumes, and indirect hemagglutination titers for 19 chickens tested for their suitability as erythrocyte donors are given (table 1). Erythrocytes from chickens 1 through 11 gave uniform serologic results, and the washed cells sedimented to form complete buttons in 28 to 44 minutes. In contrast, erythrocytes from chickens 15 through 19 had abnormally high titers and delayed sedimentation rates. The nonsensitized washed cells from chickens 15 through 19 failed to button even when held for 48 hours. The apparent ability of some abnormal erythrocytes (chickens 17–19) to sediment after sensitization with antigen might account for their not being detected by using the usual sensitized cell control. In addition to these 5 unsatisfactory donors, 3 others (chickens 12–14) had erythrocytes which could make the interpretation of results difficult.

Selection of Antigen Strains.—The antigens used to sensitize the erythrocytes in the previous study were chosen arbitrarily. In an attempt to minimize the duplication of individual antigens, polyvalent antigen preparations were made from the fewest numbers of species which would give the greatest number of somatic antigens com-

TABLE 2—Determination of Specific and Nonspecific Titers for the Selection of Antigen Strains for Salmonella Serogroups B and D

Species	Antigen strains	Somatic antigen	Indirect hemagglutinin titers		
			Positive (O antibody)	Negative	
				Range (10 serums)	Geometric mean
GROUP B			(4, 5, 12)*		
S. typhimurium	2015		1,600	0–20	6
S. typhimurium	2606		1,600	0–40	6
S. typhimurium	2014	1, 4, 5, 12	1,600	0–80	7
S. typhimurium	Ed. Ag.		800	0–10	2
GROUP D			(9, 12)*		
S. pullorum	586		800	0–80	11
S. pullorum	587	9, 12	800	0–80	12
S. pullorum	2013		800	0–80	14
S. gallinarum	1456	1, 9, 12	800	0–80	13

* Antibodies present in group-specific antiserums.

TABLE 3—Determination of Specific and Nonspecific Titers for the Selection of Antigen Strains for Salmonella Serogroup C

Antigen strains			Indirect hemagglutinin titers		
				Negative	
Species		Somatic antigen	Positive (O antibody)	Range (10 serums)	Geometric mean
SUBGROUP C_1			(6, 7)*		
S. tennesee	2978		1,600	0–40	11
S. infantis	H611		1,600	0–80	16
S.montevideo	Ed Ag.		1,600	0–80	19
S.montevideo	3928	6, 7	1,600	10–80	26
S. infantis	2975		1,600	10–160	32
S. bareilly	5628		1,600	40–160	88
S. infantis	2981		400	0–80	18
SUBGROUP C_2			(6, 8)*		
S. newport	Ed. Ag.		3,200	0–40	9
S. muenchen	2018		1,600	0–40	9
S. muenchen	5630	6, 8	3,200	0–80	12
S. muenchen	2020		3,200	0–40	14
S. muenchen	5148		1,600	0–80	14

* Antibodies present in group-specific antiserums.

mon to the frequently occurring organisms in serogroups B, C, D, and E. Duplication of antigens increased the titers obtained with some antigen strains but not with others. Certain antigen strains also appeared to give a high proportion of nonspecific reactions. In an attempt to overcome these discrepancies, erythrocytes sensitized by different species and strains of the same species were tested against group-specific and negative serums in order to select the antigen strain which gave the highest specific and lowest nonspecific titers for each serogroup. Results for members of serogroups B, C, D, and E are given (tables 2–4).

There was little difference among strains in the B and D groups. Variations within the group-specific and nonspecific titers were small. Greater differences between strains were observed in the C group which is known for its problems with false positive reactions. In the C_1 subgroup. six of the seven strains gave identical positive titers but varied greatly in negative titers. *Salmonella tennesee* gave the lowest and *Salmonella bareilly* the highest nonspecific titers. One strain of *Salmonella infantis* gave a low specific titer. In contrast to the C_1 subgroup, the cultures of the C_2 subgroup were quite similar in their reactions. Those of the E group generally had high nonspecific titers. The two *Salmonella newington* isolates gave the lowest specific and highest nonspecific titers.

In the selection of antigen strains, it may be more important to select one member of the serogroup rather than to select one for each subgroup. This is illustrated by table 5, which gives the cross reactions between common antigens and antibodies in subgroups E_1, E_2, and E_4 of serogroup E. The highest titer for each of the antigens was obtained with the specific subgroup anti-

TABLE 4—Determination of Specific and Nonspecific Titers for the Selection of Antigen Strains for Salmonella Serogroup E

Antigen strains			Indirect hemagglutinin titers		
				Negative	
Species		Somatic antigen	Positive (O antibody)	Range (10 serums)	Geometric mean
SUBGROUP E_1			(3, 10, 15)*		
S. anatum	2021		1,600	20–160	60
S. anatum	Ed. Ag.	3, 10	1,600	20–160	60
S. anatum	5138		1,600	40–160	76
S. anatum	2607		800	20–160	48
SUBGROUP E_2			(3, 10, 15)*		
S. newington	Ed. Ag.	3, 15	400	10–320	38
S. newington	H313		400	20–320	52
SUBGROUP E_4			(3, 10, 15)*		
S. senftenberg	5140	1, 3, 19	800	20–160	52
S. senftenberg	5139		800	20–160	64
S. senftenberg	Ed. Ag.		1,600	20–320	64

* Antibodies present in group-specific antiserums.

TABLE 5—Variation in Hemagglutinating Capacity of Individual Salmonella Species as Shown by a Comparison of Cross Reactions Among Common Antigens and Antibodies of the Subgroups in Serogroup E

	Hemagglutinin titers*			
	Positive serums			
Monovalent antigens	*S. anatum* 3, 10	*S. newington* 3, 15	*S. senftenberg* 1, 3, 14	Negative control
S. anatum (E_1)	12,288	10,240	6,656	80
S. newington (E_2)	10,240	15,360	1,664	100
S. senftenberg (E_4)	1,088	640	1,280	93

* Pooled data.

serum. However, the highest titers obtained with the subgroup antiserums were not necessarily given by the homologous antigen preparation. The *Salmonella senftenberg* antigen was quite poor and gave low titers with all three serums. The *S. newington* antigen gave high titers with the E_1 (10,240) and E_2 (15,360) serums but not with the E_4 (1,664) serum. The *Salmonella anatum* antigen gave high titers for all three subgroup antiserums in serogroup E and appeared to be the antigen of choice to represent the E group in a polyvalent preparation.

Stabilization of Sensitized Erythrocytes.—Sensitized chicken erythrocytes prepared for the indirect hemagglutination test lyse after four days in the refrigerator. Stable cells with a longer storage life would greatly increase the usefulness of the test. The first attempts to sensitize a stable preparation of erythrocytes made use of Cappel's stabilized 5 per cent chicken red blood cells.* A number of attempts to sensitize the cells in the presence of the original suspending fluid or after triple washing with buffer failed to yield a suitable preparation.

Since commercially stabilized cells were unsuitable for this test, stable cells were prepared according to the procedure of McKenna,[3] except that chicken rather than sheep cells were used. The formalin-preserved erythrocytes were treated to remove excess formaldehyde before they were sensitized. This time-consuming procedure (14 days) produced sensitized cells that were slow to sediment and which formed a halo rather than a compact button.

Although formalin is a good preservative for erythrocytes, it can interfere with their sensitization. In an attempt to overcome the need for the removal of excess formaldehyde, the cells were sensitized before formalin preservation. After sensitization with *Salmonella typhimurium*, the cells were thoroughly washed and the volume of the suspension was brought up to 4.5 per cent of the original volume. Formalin equal to 0.5 per cent of the original volume was then added to yield a 10 per cent suspension of sensitized cells in approximately 4 per cent formaldehyde. This 20 X stock suspension was diluted 1:20 in buffer to yield a 0.5 per cent suspension for the test. Results on the stability and sensitivity of this preparation are given (table 6). Titers

TABLE 6—Effect of Age on the Stability and Sensitivity of Salmonella typhimurium Sensitized Erythrocytes After Formalin Treatment

	Hemagglutinin titers			
Age of cells* (days)	Positive serums		Negative serums	
O (pre-formalin treatment)	320	1,280	....	40
O (post-formalinization	320	1,280	....	40
3	320	1,280	....	40
10	640	1,280	....	40
30	640	1,280	10	20
180	640	1,280	....	40
370	320	1,280	40	80

* Held at 5 C. in a refrigerator.

obtained with nontreated and three-hour formalized cells indicated that this procedure had not altered the sensitivity of the erythrocyte preparation. The stability of these formalized cells, without a loss of sensitivity, was indicated by the similar titers obtained after three, ten, 30, and 180 days storage at 5 C. There was a slight tendency toward nonspecific reactions after 12 months.

In order to observe the effect of holding temperature on the stability and sensitivity of the formalin-stabilized cells, aliquots of a preparation were maintained at different temperatures for one week. The indirect hemagglutinin titers obtained with the different aliquots after holding and the condition of the cell control are given (table 7).

* Baltimore Biological Laboratory, Inc., Baltimore, Md.

The samples held at room temperature (20 C.) and in the clinician's truck (10 to 30 C.) for one week gave titers similar to those of the refrigerated (5 C.) control. Holding at 38 C. apparently had an adverse effect upon the cells, which was indicated by the increase in nonspecific titers of the negative serums. The sample held at 48 C. was destroyed by cell lysis. Temperatures above 20 C. impaired the ability of the cells to form a compact button.

TABLE 7—The Effect of Holding Temperature on the Stability and Sensitivity of Formalin Stabilized Salmonella typhimurium Sensitized Erythrocytes

7-Day holding temperature*	Cell control	Hemagglutinin titers			
		Positive serums		Negative serums	
5 C.	Good button	320	1,280	10	10
20 C.	Good button	320	1,280	20	10
38 C.	Fair button	320	1,280	80	80
48 C.	No button	>2,560	>2,560	>360	>360
10–30 C.**	Fair button	320	1,280	20	10

* Six-month-old material at 5 C. ** Material kept in truck subject to temperature fluctuation.

DISCUSSION

The indirect hemagglutination system has been used as a polyvalent test for the detection of salmonellosis in both man [4] and poultry.[6-8] The reason why this test has remained essentially a research rather than a diagnostic tool is probably due to the four-day refrigerator life of sensitized cells. The fact that sensitized cells which have been formalized maintain their sensitivity for 180 to 370 days should make the results more uniform and the test more useful. The ability of formalized preparations to withstand moderate temperatures (20 to 30 C.) for up to seven days should permit their use in the field.

In addition to instability, two other possible objections to the test have been recognized and remedied. One is the false titers given by erythrocyte preparations from certain birds. Although the sensitized cells from these birds may sediment within an hour, their abnormality can be observed by the failure of washed, nonsensitized cells to sediment and form a compact button. There appears to be a correlation of this abnormality with the occurrence of the lesions of the avian leukosis complex. A possible explanation for the failure of washed cells from leukotic birds to sediment is that virus particles adsorbed to the surface of erythrocytes may cause their agglutination. The presence of blood fluids in unwashed erythrocyte suspensions and Salmonella antigens in sensitized preparations may prevent the agglutination of leukotic erythrocytes by virus particles. This apparent correlation of autoagglutination by washed erythrocytes and the lesions of the avian leukosis complex should be studied further.

The judicious selection of antigen strains can minimize the occurrence of false titers as well as increase the sensitivity of the test. Although the number of antigen strains tested was small, certain trends were quite apparent. Certain species, such as *S. typhimurium*, gave uniform results; others, e.g., *S. infantis*, varied greatly in their hemagglutination properties. The selection of antigen strains on the basis of those yielding the highest specific and lowest nonspecific titers appears to be adequate.

SUMMARY

In a study designed to improve the reliability and storage quality of chicken erythrocytes sensitized with Salmonella antigens for the polyvalent indirect hemagglutination test, procedures were developed to detect abnormal blood cells, select optimum antigen strains, and stabilize the sensitized erythrocyte preparations. Red blood cells from certain birds which did not yield an end point or gave false high titers, could be detected by their prolonged sedimentation rate after washing but before sensitization. There was some correlation between this phenomenon and the lesions of the avian leukosis complex. Suitable antigen strains were selected on the basis of those giving low nonspecific titers while still giving high specific titers. The addition of formalin to 10 per cent suspensions of sensitized erythrocytes yielded cells which were stable for at least seven days at 20 C. and 180 days at 5 C. The preparation of standardized, sensitized erythrocytes that

are stable up to one year should increase the accuracy and application of the indirect hemagglutination test.

References

[1] Keogh, E. V., North, E. A., and Warburton, M. F.: Hemagglutinins of the Hemophilus Group. Nature, 160, (1947): 63.

[2] Keogh, E. V., North, E. A., and Warburton, M. F.: Adsorption of Bacterial Polysaccharide to Erythrocytes. Nature, 161, (1948): 687–688.

[3] McKenna, J. M.: A Stable Preparation of Antigen-Sensitized Erythrocytes. Proc. Soc. Exptl. Biol. & Med., 95, (1957): 413–416.

[4] Neter, E.: Bacterial Hemagglutination and Hemolysis. Bact. Rev., 20, (1956): 166–188.

[5] Neter, E., Grozynski, E. A., Gino, R. M., Westphal, O., and Luderitz, O.: The Enterobacterial Hemagglutination Test and Its Diagnostic Potentialities. Canad. J. Microbiol., 2, (1956): 232–244.

[6] Sieburth, J. McN.: Indirect Hemagglutination Studies on Salmonellosis of Chickens. J. Immunol., 78, (1957): 380–386.

[7] Sieburth, J. McN.: The Effect of Furazolidone on the Cultural and Serological Response of *Salmonella typhimurium* Infected Chickens. Avial Dis., 1, (1957): 180–194.

[8] Sieburth, J. McN.: The Indirect Hemagglutination Test in the Avian Salmonella Problem. Am. J. Vet. Res., 19, (July, 1958): 729–735.

Summario in Interlingua

Stabile, Standardisate, Sensibilisate Erythrocytos de Gallina pro le Polyvalente Indirecte Test de Hemagglutination a Salmonella

In un studio visante a meliorar le fidelitate e le qualitates immagasinatori de erythrocytos de gallina sensibilisate con antigenos de Salmonella pro le polyvalente indirecte test de hemagglutination, un procedimento esseva disveloppate pro deteger cellulas sanguinee anormal, pro seliger racias de alte qualitate antigenic, e pro stabilisar le sensibilisate preparatos erythrocytic. Erythrocytos ab certe aves que non effectuava un puncto terminal o resultava in titros falsemente elevate poteva esser detegite per lor prolongate sedimentation post le lavation sed ante le sensibilisation. Esseva notate un certe correlation inter iste phenomeno e le lesiones del complexo de leucosis avian. Racias del desirate qualitate antigenic esseva seligite secundo le criterio que illos debeva distinguer se per basse titros nonspecific durante que le titros specific esseva alte. Le addition de formalina a suspensiones de 10 pro cento de sensibilisate erythrocytos resultava in un stabilitate del cellulas durante al minus septe dies a 20 C e durante 180 dies a 5 C. Le preparation de standardisate, sensibilisate erythrocytos con un stabilitate durante usque a un anno resultarea certo in un plus alte accuratia e un plus extense applicabilitate del indirecte test de hemagglutination.

Vital Staining and Differentiation of Microfilariae

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IT WAS RECENTLY REPORTED [8] that microfilariae of *Dirofilaria immitis* and Dipetalonema sp., vitally stained with acridine orange, could be examined in thick blood film preparations by means of dark ground illumination with glass or gelatin color filters. Since both forms appeared to be stained almost identically, no attempts were made to identify possible differences microscopically.

After investigating the effects of a number of vital stains, microfilariae of *D. immitis*, Dipetalonema sp., and *Dirofilaria uniformis* were found to react differently to Coriphosphine O (C.I. 787) so that differentiation of the viable microfilariae was possible. This compound is closely related to acridine orange. In surveying canine populations, the acridine orange technique [8] supplemented by the differential staining obtained with Coriphosphine O was found to be as accurate and as rapid for the differentiation of microfilariae as the concentration method originally described by Knott [3] and modified by Newton and Wright.[4,5]

MATERIALS AND METHODS

Samples, positive for microfilariae by the acridine orange method, were tested by adding 1 volume of solution (100 mg./100 ml.) of Coriphosphine O (Chroma or National aniline) in distilled water to 9 volumes of blood containing 20 USP units of heparin per milliliter. After the samples were mixed several times with a pipette, the mixture was allowed to stand at room temperature for about five minutes. A wet, coverglass mount was made from approximately 20 to 40 cmm. of the mixture, and this preparation was examined as previously reported by Rothstein.[8] A Corning No. 5030 filter was used at the light source because it permitted much more light to pass through than the Wrattan C5 filter used previously. A Corning No. 3486 yellow filter was often used in the ocular when very thick blood preparations were examined.

Aliquots of the heparinized blood samples tested by the vital staining technique were also subjected to the 2 per cent formalin and methylene blue concentration method. Heparin was chosen as an anticoagulant because oxalate killed the microfilariae with in 24 to 48 hours at room temperature. Blood samples used in this study were obtained from dogs purchased by this Institute for use in research investigation. Rabbit blood containing microfilariae of *D. uniformis* was also examined under similar conditions.

RESULTS

A wet film preparation of canine blood containing microfilariae of *D. immitis* stained with Coriphosphine O (final concentration of 1:10,000) was examined at x75 magnification five to ten minutes after the dye had been added. The microfilariae remained viable and appeared to be distinctly stained only at their extremities (fig. 1, 2). When the preparations were examined within five minutes after addition of the dye, only the extreme anterior somatic nucleus appeared to have taken up the dye in some individuals. In other microfilariae, only the posterior terminal nuclei were stained (fig. 3). This selective cytochemical differentiation persisted for as long as three hours. At the end of that time, however, x 430 magnification disclosed that the central portion of the microfilaria was beginning to absorb the dye. At the end of six hours, many of the microfilariae were dead. Approximately 25 per cent of the organisms were completely stained; another 25 per cent were stained only in the extremities; and the remaining 50 per cent were stained heavily in the extremities and lightly in the central portions of the parasites.

When blood containing microfilariae of the Dipetalonema sp. was stained in the same manner, the results were quite different. No microfilariae were stained after ten minutes and nearly all remained unstained after 30 minutes. One hour after the dye had been added, however, the microfilariae were stained completely. The cytochemical gradient response seen in the reaction of

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The authors thank Captain Arthur P. Moon and Mr. Ralph Duxbury, Department of Medical Zoology, for supplying the blood samples containing *D. uniformis* microfilariae; Dr. Frank B. Johnson for supplying the Chroma Coriphosphine O; Dr. Benjamin H. Bruckner, chief, Department of Veterinary Chemistry, for support during this investigation; and Miss Sally Craig, Medical Audio-Visual Department, for the black-and-white photographic reproductions.

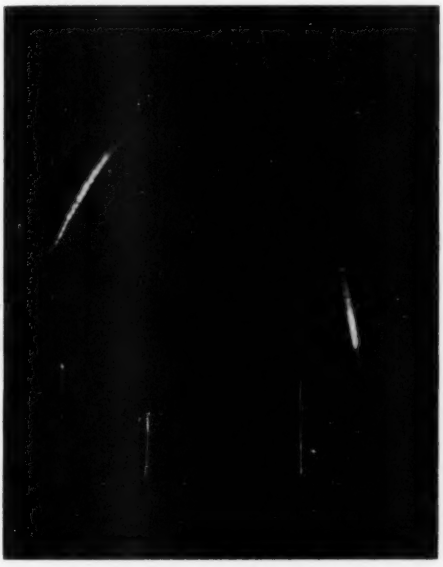

Fig. 1—Microfilaria of Dirofilaria immitis after staining with a 1:10,000 dilution of Coriphosphine O for 15 minutes. x 287.

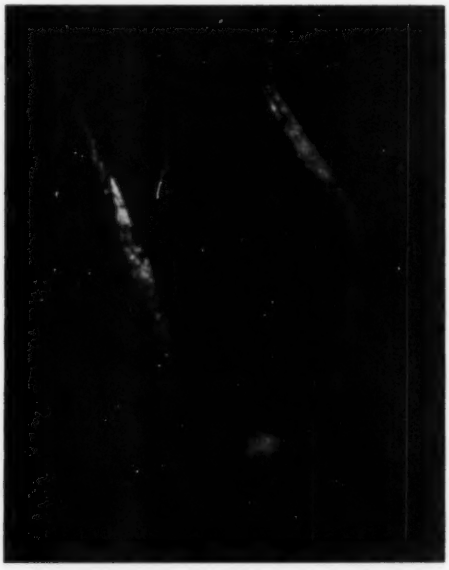

Fig. 2—Microfilaria of Dirofilaria imitis after staining with 1:10,000 dilution of Coriphosphine O for 30 minutes. x 573.

the microfilariae of *D. immitis* with Coriphosphine O was absent in the microfilariae of Dipetalonema.

Microfilariae of *D. uniformis* took up no dye within five minutes after its addition, but a bright, bulging, optically-refractive spot, 95 to 100 μ from the anterior margin of the sheath, was distinctly visible (fig. 4, 5). After about 15 minutes of exposure, this spot was seen to be about 25 μ posterior to the nerve ring. The microfilariae were motile, but sluggish. Within 30 minutes, no moving forms were seen on the slide. The refractile spot was very prominent and most of the microfilariae were stained in all areas except the central portion. A few were completely stained. When viewed in the dorsoventral aspect, the refractile body appeared as a bulge containing a bright yellow somatic nucleus situated in a depression. It was evident that the forms which did not have this appearance were in a position where such a structure was not visible. After 20 hours at room temperature, microfilariae of *D. uniformis* in the heparinized blood sample without dye were no longer motile, differing in this respect from *D immitis* microfilariae, which stayed alive for ten to 19 days. The nonmotile forms of *D. uniformis* were not stained with Cori-

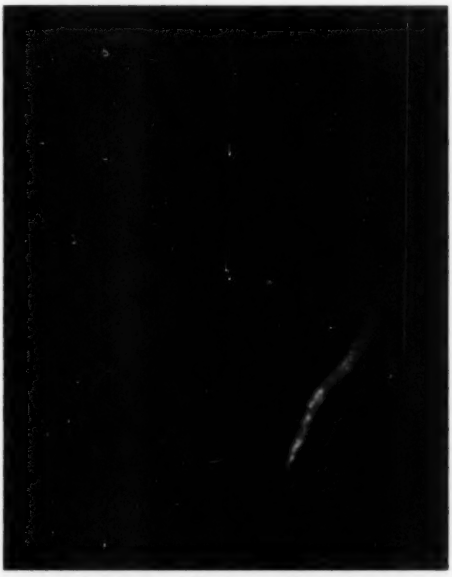

Fig. 3—Microfilaria of Dirofilaria immitis after staining with a 1:10,000 dilution of Coriphosphine O for 10 minutes. x 477.

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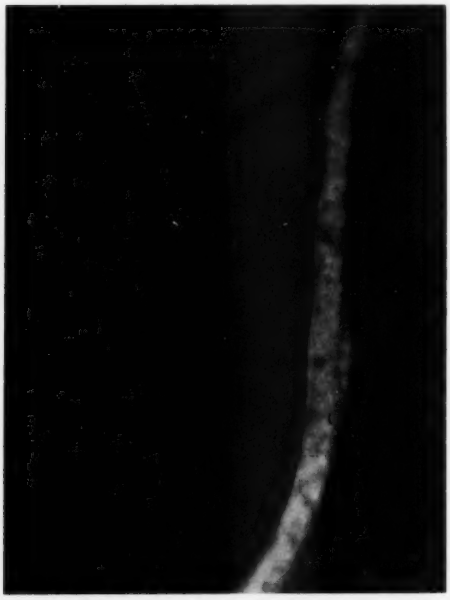

Fig. 4—Microfilaria of Dirofilaria uniformis after staining with a 1:10,000 dilution of Coriphosphine O for five minutes. x 753.

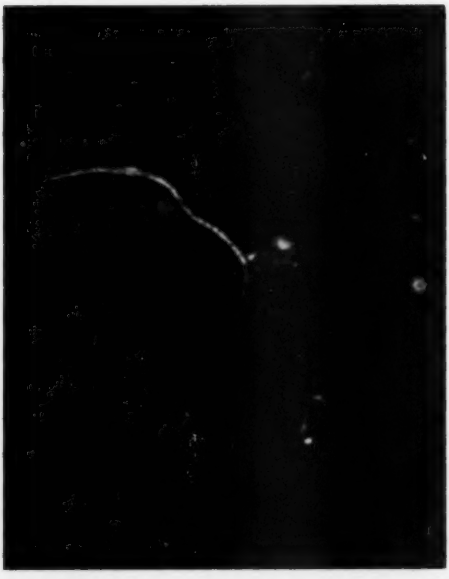

Fig. 5—Microfilaria of Dirofilaria uniformis after staining with a 1:10,000 dilution of Coriphosphine O for five minutes. x 147.

phosphine O within five minutes, but the refractile body was still visible. Within 15 minutes, all the microfilariae were completely stained.

When microfilariae of *D. uniformis* were exposed to a final concentration of 1:10,000 of acridine orange, all organisms were uniformly and completely stained within five minutes.

The 282 canine blood samples which had been examined by the concentration technique were also tested by this vital staining method. Of the 73 positive identifications made by either technique or by both, the vital staining method using acridine orange detected 69 positive blood samples and missed four which were detected by the concentration method. The modified Knott technique detected 61 positive samples and missed 12 which were detected by the vital staining technique. A statistical analysis based on chi-square, with the level of significance of 0.05, indicated that the vital staining technique is capable of detecting more positive blood samples than the modified Knott method.

DISCUSSION

With the proven existence of at least two types of microfilariae in the peripheral blood of dogs in this country, it is important to differentiate the microfilariae detected in blood specimens obtained from the dog. Otto and Bauman [6] believe that microfilariae are most easily differentiated when they are killed and stained in an extended position, as they are said to be in the modified Knott method. Using this technique, Newton and Wright found that over 90 per cent of the specimens of Dipetalonema microfilariae ended in a buttonhook, while the tails of the microfilariae of *D. immitis* ended in a straight line. During this experiment, preparations were observed in which almost 50 per cent of the Dipetalonema forms did not have the typical buttonhook appearance of the tails. Often, the tails were seen to end in "V" bends of varying angles. Similarly, microfilariae of *D. immitis* were observed which did not end in a straight line but in kinks or bends of varying degrees.

Microfilariae in the peripheral blood of dogs and rabbits were easily detected by the vital staining technique described, using acridine orange as the preferred fluorochrome.[8] With colored glass filters as ancillary aids in the dark field examination

of whole blood, thick preparations of blood no longer presented a problem in scanning for blood parasites. Multiple layers of erythrocytes did not interfere in rapid microscopic examination for the stained viable parasites. The need for laking the erythrocytes or for concentrating the parasites by centrifugation was eliminated.

The motility of the stained microfilariae aided considerably in the detection of the organisms. Distinctive movements of the two types of microfilariae under these conditions seemed to offer a more dependable criterion for differentiation than did the appearance of the terminal portions of the fixed forms. The movement of *D. immitis* microfilariae was generally of the undulating type. At a given moment, the body had at least two smooth curves along the length of the parasite. The movement generally seen in the Dipetalonema forms was rather erratic; sometimes the movement followed the pattern of a coiled or compressed spring, and often one or two knot-like formations occurred along the length of the microfilariae.

Another observation made with this technique was the consistent difference in the number of microfilariae observed. In the case of Dipetalonema infections, rarely were more than 3 or 4 microfilariae observed per 20 to 40 cmm. of blood. As many as 50 to 65, and never less than 3 to 5, microfilariae of *D. immitis* were seen on a slide prepared in the same manner.

After detecting the presence of microfilariae with acridine orange, the addition of Coriphosphine O to an aliquot of the blood sample definitely differentiated the microfilariae within five to ten minutes after the addition of the second dye. The microfilariae of *D. immitis* were motile and appeared distinctly stained only in the extremities. This selective histochemical differentiation persisted for as long as three hours. In contrast, the microfilariae of Dipetalonema sp. did not take up the stain for at least 30 minutes to one hour, after which they usually became completely stained.

It is interesting to speculate about the fundamental basis for the cytochemical differences observed. These may imply that a physiologic and morphologic differentiation exists between microfilariae of *D. immitis* and Dipetalonema.

The results obtained also suggest that there is a cytologic gradient response with respect to dye uptake on the part of *D. immitis* microfilariae only, when both types are stained with Coriphosphine O as described. In addition, the reaction of *D. immitis* microfilariae demonstrated that the thresholds for acridine orange and Coriphosphine O were quite different. When stained with acridine orange, the microfilariae stayed alive at room temperature for at least ten to 19 days. Under these conditions, they underwent *in vitro* development ordinarily seen only in the mosquito, as described by Feng,[2] Webber,[10] and Pistey,[7] and in the waxmoth larva, an abnormal host, described by Yoeli.[11] In contrast, when stained with Coriphosphine O, living forms were seldom seen on slides for much longer than six hours, or for more than 24 hours when kept in test tubes at room temperature.

In addition to affording opportunities of studying *in vitro* development, the vital staining technique has the additional advantage of permitting the investigator to see the effects of various external factors, such as dyes, on the microfilariae. Host-parasite relationships may also be studied with relative ease and in a microcosm where the parasite is still living.

The authors believe that this method of dye-differentiation should be used to re-examine the differentiation of microfilariae accomplished by previous investigators. Faust's method [1] of expressing the relative distances of the four major internal structures of microfilariae with respect to one another along the longitudinal axis has long been the basis for differentiation of the seven species of microfilariae found in man. In a survey of microfilarial populations of birds, Saunders [9] revealed what appeared to be nine different microfilarial forms. By statistical treatment of the data obtained with Faust's technique, she showed the probability that there were eight types of microfilariae. In view of the fact that only probable differentiation of microfilariae was obtained from thin films that were air-dried and alcohol-fixed prior to Giemsa staining, it is important to apply the vital staining technique to microfilarial forms that parasitize man and animals.

SUMMARY

Viable microfilariae of Dipetalonema sp. and *Dirofilaria immitis* in the peripheral blood of dogs and of *D. uniformis* in the

rabbit were easily detected by the vital staining technique described, using acridine orange as the preferred fluorochrome. Distinctive differences in the movement of the canine types were seen. The movement of *D. immitis* microfilariae was generally of the undulating type, in contrast to the erratic movement of the Dipetalonema forms. Another consistent difference noticed was in the number of microfilariae observed. In Dipetalonema infections, rarely were more than 3 or 4 microfilariae observed per 20 to 40 cmm. of blood. As many as 50 to 65, and never less than 3 to 5, microfilariae of *D. immitis* were seen on a slide prepared in the same manner. Further differentiation was accomplished with the closely related dye, Coriphosphine O. Within ten minutes, microfilariae of *D. immitis* were stained only at their extremities, while an interval of 30 minutes or more was required before those of Dipetalonema stained, not in the extremities but throughout their entire forms. A comparative study of 282 canine blood samples indicated that use of the vital staining technique detected more positive blood samples than the modified Knott method.

References

[1] Faust, E. C.: Human Helminthology. 3rd ed. Lea & Febiger, Philadelphia, 1949.

[2] Feng, L. C.: Development of *Microfilaria malayi* in *A. hyrcanus* var. *Sinensis* Wied. Chinese M. J., I, (1936): 345–367.

[3] Knott, J.: A Method for Making Microfilarial Surveys on Dog Blood. Tr. Roy. Soc. Trop. Med. & Hyg., 33, (1939): 191–196.

[4] Newton, W. L., and Wright, W. H.: The Occurrence of a Dog Filarrid Other Than *Dirofilaria immitis* in the United States. J. Parsitol., 42, (1956): 246–258.

[5] Newton, W. L., and Wright, W. H.: A Reevaluation of the Canine Filariasis Problem in the United States. Vet. Med., 52, (1959): 75–78.

[6] Otto, G. F., and Bauman, P. M.: Canine Filariasis. Vet. Med., 54, (1959): 87–96.

[7] Pistey, W. R.: Studies on the Development of *Dirofilaria tenuis*, Chandler 1942. J. Parasitol., 44, (1958): 613–626.

[8] Rothstein, N.: Vital Staining of Blood Parasites with Acridine Orange. J. Parasitol., 44, (1958): 588–596.

[9] Saunders, D. C.: Microfilariae and Other Blood Parasites in Mexican Wild Doves and Pigeons. J. Parasitol., 45, (1959): 69–75.

[10] Webber, W. A. F.: *Dirofilaria aethiops* Webber, 1955, A Filarial Parasite of Monkeys: III. The Larval Development in Mosquitoes. Parasitology, 45, (1955): 388–400.

[11] Yoeli, M., Alger, N., and Most, H.: Studies on Filariasis. I. The Behavior of Microfilariae of *Dirofilaria immitis* in the Wax Moth Larva *(Galleria mellonella)*. Exptl. Parasitol., 7, (1958): 531–541.

SUMMARIO IN INTERLINGUA

Le Tincturation Vital e le Differentiation de Microfilarias

Viabile microfilarias de Dipetalonema sp. e de *Dirofilaria immitis* in le sanguine peripheric del can e de *D. uniformis* in le conilio esseva facilemente detegite per un technica de tincturation vital que utilisa orange acridinic como fluorochromo preferite. Le technica es describite in detalio. Esseva constatate distinctive differentias in le movimento del typos canin. Le movimento de microfilarias de *D. immitis* esseva generalmente de typo undiforme, per contrasto con le movimento erratic del microfilarias de Dipetalonema. Un altere constante differentia notate concerne le numero del microfilarias observate. In infectiones dipetalonematose, le constatation de plus que 3 o 4 microfilarias in 20 a 40 mm^3 de sanguine esseva rar. In le caso de *D. immitis*, le mesme typo de preparato monstrava usque a 50 o 65 e nunquam minus que 3 a 5 microfilarias. Un differentiation additional esseva effectuate per medio del intimemente relationate colorante, Coriphosphina O. Intra dece minutas, microfilarias de *D. immitis* esseva tincturate solmente a lor extremitate, durante que un intervallo de 30 minutas o plus esseva requirite pro le tincturation de microfilarias de Dipetalonema, e iste tincturation non se initiava al extremitate sed afficeva le forma total. Un studio comparative de 282 specimens de sanguine canin indicava que le technica del tincturation vital resulta in le detection de un plus alte numero de casos positive que le modificate methodo de Knott.

Seromucoid Levels in Normal and Hospitalized Domestic and Exotic Animal Species

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Davis and San Diego, California

THE EXISTENCE of highly soluble carbohydrate-protein conjugates in mammalian serum was reported by Freund, in 1892,[8] Zanetti, in 1897,[28] and Bywater, in 1909.[3] This carbohydrate-rich heterogeneous protein fraction, which they isolated from serum deproteinized by heat, was named seromucoid due to its similarity to ovomucoid.[28] The seromucoid fraction was found to contain about 25 per cent carbohydrate following hydrolysis and, in man, accounts for approximately 10 per cent of the total protein-bound hexose in the serum.[23] In man, similar fractions have been isolated by various techniques using filtrates of serum or plasma deproteinated with perchloric acid [24] and sulfosalicylic acid, or both. Winzler [24] recently devised a method for the estimation of the seromucoid level by precipitation with 5 per cent phosphotungstic acid in 2 N HCl following initial deproteinization by 0.6 M perchloric acid. The seromucoid can then be measured in terms of its protein, tyrosine, hexose, hexosamine, or sialic acid content.

Carbohydrate-protein complexes in the serum can be divided into two groups, glycoproteins and mucoproteins.[26] Most of the globulins apparently fit into the glycoprotein category, with the carbohydrate units linked to amino acids by stable chemical bonds which require degradation of the entire molecule for their individual separation. Glycoids are glycoproteins containing less than 4 per cent hexosamine, while mucoids are those containing greater than 4 per cent hexosamine. Seromucoid belongs to the group of mucoids.

Mucoproteins are composed of acid mucopolysaccharides, such as hyaluronic acid, chondroitin sulfates, and heparin, which are linked to the protein portion of the molecule by either a hydrogen or salt bond. These linkages are readily split. The acid mucopolysaccharides of connective tissue contain uronic acid; the seromucoid and other serum glycoproteins do not. Much confusion has arisen due to inconsistencies in terminology and misinterpretations of findings.

A great variety of pathologic conditions, such as infectious diseases, surgical interventions, malignancies, and some metabolic diseases, cause significant increases in the seromucoid level in man.[25] Marked increases have been observed in the serum of human beings with tuberculosis, typhoid fever, rheumatoid arthritis, uncontrolled diabetes mellitus, myxedema, and some malignant tumors.[18] In man, diffuse parenchymatous hepatic disease without complications of infection or neoplasia may result in a fall of the seromucoid level to 50 per cent of its usual level, since the main site of its production is the liver.[23] However, finding increased serum glycoproteins in pathologic serums is strong evidence that some of the carbohydrate-protein complexes may originate from damaged tissues. It has been proposed that the depolymerization of the ground substance of the connective tissue in degenerative or proliferative processes may release solubilized components to the serum. The high levels of glycoproteins that result from the administration of parathyroid extract would also suggest an extrahepatic origin of certain constituents within this heterogeneous fraction.[10, 11] Of special interest is the controversial relationship of the adrenal cortical hormones to the serum protein-bound polysaccharide levels. It has been reported that corticotropin administration may produce an increase in the seromucoid level in patients with acute rheumatic fever but does not significantly elevate the seromucoid levels in normal guinea pigs.[23] Rabbits given DDD (2, 2-bis-[para-chlorphenyl]-1, 1-dichloroethane), which produces a degeneration of the zona fasciculata of the adrenal cortex, were shown to have significant decreases of the blood polysaccharide level.[20] Boas and Peterman [1] have postulated that elevated serum protein-bound hexose may represent a systemic response to nonspecific stress.

A few limited investigations on the plasma glycoproteins in domestic animals have been reported. Mayer, in 1942,[19] analyzed a glycoprotein fraction isolated from horse serum with 60 per cent ethanol following initial deproteinization. This fraction was present in a concentration of 10.1 mg./100 cc. of serum and contained nitrogen (Kjeldahl), 10.85 per cent; carbohydrate, 18.87 per cent; glucosamine, 10.58 per cent; and sulfur, 3.98 per cent. Weimar and Winzler [22] have isolated and analyzed an α-1 acidic glycoprotein, called orosomucoid, from pooled ox and horse serum; this is

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TABLE 1—Plasma Seromucoid Levels in Various Domestic Animals

Species	Sex	Age	Protein (mg./100 ml.) n*	$\bar{x}$**	σ†	Protein-bound sialic acid (mg./100 ml.) n	$\bar{x}$	σ	Protein/sialic acid ratio n	$\bar{x}$	σ
Equine	M & F	1 yr.	87	21.5 ± 7.36		51	1.69 ± 0.32		47	11.2 ± 2.57	
Bovine	Lactating dairy cows	2 yr.	19	41.3 ± 15.7		....	....		....	....	
Bovine	Bulls and steers	1 yr.	7	47.8 ± 9.93		7	2.87 ± 0.45		7	16.7 ± 3.07	
Bovine	Nonlactating cows	1 yr.	16	25.9 ± 12.5		16	1.86 ± 0.53		16	13.5 ± 3.22	
Ovine	M & F	6 mo.	37	33.4 ± 15.8		35	2.12 ± 0.76		35	15.2 ± 5.31	
Porcine	M & F	3 mo.	5	112.2 ± 27.4		5	5.51 ± 1.07		5	20.3 ± 3.12	
Caprine	M & F	6 mo.	5	32.3 ± 15.5		5	2.26 ± 0.70		5	13.9 ± 2.60	

* n = number of animals. ** $\bar{x}$ = mean. † σ = standard deviation.

the major constituent of the seromucoid fraction in man. Cornelius *et al.*[5] reported an increase in both the seromucoid fraction and the urinary glycoproteins of sheep with experimental urolithiasis. Tadati[19] recently reported that dogs affected by various skin diseases had increases of this blood fraction. Investigations on serums from newborn and fetal sheep and calves have revealed relatively high amounts of a glycoprotein called fetuin, which later disappears.[14] Fetuin was found to contain 5.3 per cent hexose, 9.9 per cent hexosamine, and 6.0 per cent sialic acid (neuraminic acid) and its presence may reflect the hyperplasia of embryonic development. Elevations in the serum polysaccharide levels have been observed in dogs after the production of bacterial or sterile abscesses and extensive surgery.[17]

TABLE 2—Partial Composition (%) of Plasma Seromucoid Fraction in the Horse, Cow, and Sheep

Species	Age	Protein	Hexose	Hexosamine	Sialic acid
Horse 59-1	Mature	61	8.0	5.8	5.2
Horse 59-2	Mature	73	6.2	4.5	4.6
Cow 347	Mature	61	7.6	7.1	4.9
Cow 396	Mature	67	8.4	5.4	3.8
Sheep 59-1	6 mo.	65	8.1	7.3	4.5
Sheep 59-2	6 mo.	60	8.1	3.7	4.1

Percentages were determined on a moisture-free basis.

The purposes of this investigation were (1) To determine the normal plasma seromucoid levels in horses, cattle, swine, sheep, and goats. (2) To report the plasma seromucoid levels in hospitalized animals with various pathologic conditions. (3) To report the plasma seromucoid levels in various zoo species.

MATERIALS AND METHODS

Blood samples (50 ml.) were collected, by jugular puncture, into vials containing crystalline lithium oxalate to give a concentration of 1 mg. per milliliter. All exotic species were caged at the San Diego Zoo, and blood samples were collected there. From each animal, 20 ml. of plasma was harvested, allowing measurement of the seromucoid in terms of both its protein and protein-bound sialic acid content. The seromucoid was precipitated from plasma as described by Weimer and Moshin.[21] The seromucoid was expressed in terms of albumin-protein, using the biuret reagent,[27] and protein-bound sialic acid, by its reaction with diphenylamine as described by Winzler.[22] Standard curves were prepared, using crystalline n-acetyl sialic acid, which has been established by Martensson *et al.*[12] to occur biologically as an important chemical species of sialic acid conjugates in the serums of the horse, cow, pig, and sheep.

Seromucoid isolated from plasma of the horse, cow, sheep, and pig was analyzed for protein content by the biuret reaction,[27] hexosamine content

TABLE 3—Plasma Seromucoid Levels in Hospitalized Horses

Age (yr.)	Sex	Protein (mg./100 ml.)	Protein-bound sialic acid (mg./100 ml.)	Protein/sialic acid ratio	Clinical condition
4	F	26.5	.....	.....	Anemia with acute hepatic necrosis.
11	F	42.2	.....	.....	Subacute hepatic necrosis.
7	F	47.9	.....	.....	Bacterial pneumonia; Parascaris and strongyle infection.
9	M	25.1	1.78	14.1	Tetanus.
1	M	33.1	.....	.....	Tetanus.
10	F	22.8	1.73	13.8	Congestive heart failure.
5	...	39.9	.....	.....	Congestive heart failure.
13	M	42.2	2.73	15.5	Lymphosarcoma.
16	M	58.7	2.50	23.8	Plasma-cell myelomatosis.
11	F	38.8	.....	.....	Suppurative nephritis.
2	M	31.1	.....	.....	Undiagnosed anemia and edema (hemoglobin 4.5 Gm./100 ml.).
1	F	33.1	3.08	10.7	Star thistle poisoning with associated encephalomalacia.
12	M	18.5	.....	.....	Lymphosarcoma.

TABLE 4—Plasma Seromucoid Levels in Hospitalized Cattle

Age (yr.)	Sex	Protein (mg./100 ml.)	Protein-bound sialic acid (mg./100 ml.)	Protein/sialic acid ratio	Clinical condition
Mature	M	80.2	4.10	19.6	Postsurgical urethrotomy (following obstruction by calculi with a ruptured urinary bladder).
2	M	51.5	3.00	17.1	Urinary calculi with urethral rupture.
1	M	54.8	2.85	19.2	Postsurgical urethrotomy (following urinary obstruction from calculi).
1	M	112.3	4.70	24.0	Postsurgical urethrotomy from urinary obstruction from calculi.
Mature	F	89.7	3.21	28.0	Traumatic reticulitis.
6	F	67.8	.....	.....	Undiagnosed indigestion, possible traumatic reticulitis.
3	F	43.6	.....	.....	Indigestion.
2	F	42.9	2.88	14.9	Foundered, with fracture of tibia.
5	F	72.6	4.54	16.0	Postsurgical (rumenotomy) and pneumonia.
2	F	75.3	.....	.....	Chronic suppurative pneumonia.
Mature	F	198.5	.....	.....	Metritis following delivery of a dead fetus and obturator paralysis.
6	F	40.7	2.93	13.9	Postsurgical (eye enucleation).
Mature	F	64.7	.....	.....	Postsurgical bloat (trocharization).
1	F	136.3	.....	.....	Vaginal and rectal prolapse.
1	...	66.5	.....	.....	Chronic trichostrongyloidosis with edema.
4	F	38.4	2.89	13.3	Trichostrongyloidosis and coccidiosis.
Mature	F	51.9	2.94	17.7	Prolonged gestation.
2	M	143.0	.....	.....	Acute toxic indigestion.
14	F	42.0	.....	.....	Lymphosarcoma.
2	F	101.0	.....	.....	Lymphosarcoma with acute leukemia.

by Schloss'[16] modification of the Elson and Morgan method[7] (preceded by acid hydrolysis, as described by Boyce *et al.*[2]), sialic acid content by the diphenylamine reaction,[28] and total hexose content by the orcinol reaction.[23]

RESULTS

Normal plasma seromucoid levels for horses, lactating and nonlactating cows, bulls, steers, sheep, pigs, and goats are presented (table 1). The seromucoid is expressed in terms of both its protein and sialic acid content. A ratio of the protein to sialic acid is also expressed for each species. Plasma seromucoid levels are reported for hospitalized horses, cattle, pigs, sheep, and goats with various pathologic conditions (tables 3–5). Horses admitted to the University hospital for various abnormalities had a mean plasma seromucoid value of 25.9 ± 8.25 mg./100 ml. of blood when expressed as protein (45 horses) and 1.93 ± 0.434 mg./100 ml., expressed as protein-bound sialic acid (10 horses). The slight mean increase above the normal values in the seromucoid-protein in hospitalized horses was statistically significant ($p = < 0.01$). A partial chemical analysis of this glycoprotein fraction in the horse, cow, and sheep is presented (table 2). In cattle with inflammatory lesions from various diseases, the mean plasma seromucoid level was significantly elevated at 89.0 ± 45.4 mg. of protein (11 cattle) and 3.61 ± 0.81 mg. of protein-bound sialic acid (7 cattle).

TABLE 5—Plasma Seromucoid Levels in Hospitalized Sheep, Goats, and Pigs

No.	Species	Age	Sex	Protein (mg./100 ml.)	Protein-bound sialic acid (mg./100 mg.)	Protein/sialic acid ratio	Clinical condition
1	Ovine	6 mo.	M	149.2	7.60	19.6	Rectal prolapse.
1	Ovine	6 mo.	M	96.8	4.59	20.9	Cellulitis—forelimb.
1	Ovine	6 mo.	M	67.3	4.79	14.1	Cellulitis—forelimb.
1	Ovine	4 mo.	....	97.2	5.02	19.3	Acute coccidiosis.
1	Ovine	4 mo.	....	33.1	1.94	17.1	Subacute coccidiosis.
1	Ovine	1 yr.	F	86.4	.....	.....	Febrile—undiagnosed.
1	Ovine	4 mo.	M	94.6	.....	.....	Urethral obstruction from calculi.
1	Ovine	.....	M	65.8	.....	.....	Renal amyloidosis and trichostrongyloidosis.
6	Caprine	Mature	M & F	35.0 ± 9.4	2.31 ± 0.52	15.3 ± 2.3	Extensive verminous pneumonia (*Muellerius capillaris*).
1	Caprine	5 yr.	M	23.7	2.11	11.2	Tetanus.
4	Porcine	.....	M & F	125.8 ± 7.62	5.56 ± 1.03	23.0 ± 3.1	Mild virus pneumonia.

TABLE 6—Plasma Seromucoid Levels in Various Zoo Species Caged at the San Diego Zoo

Common name	Genus and species	Sex	Age	Protein	Protein-bound sialic acid	Protein/sialic acid ratio	Clinical condition
Alpaca	*Lama glama*, var. *pacos*	F	7 yr.	35.5	2.78	12.77	Pregnant.
Llama	*Lama guanicoe*, var. *glama*	F	M*	42.9	2.97	14.44	Pregnant.
Guanaco	*Lama guanicoe*, var. *huanachus*	F	6 mo.	65.0	4.33	15.01	Normal.
Vicuña	*Lama vicugna*, var. *mensalis*	F	M	28.3	2.65	10.68	Pregnant.
Camel	*Camelus dromedarius*	F	...	11.7	1.39	8.42	Normal.
Aoudad	*Ammotragus lervia*	F	M	36.6	2.17	16.87	Pregnant.
Big horn sheep	*Ovis canadensis*	F	M	35.5	2.53	14.03	Pregnant.
Big horn sheep	*Ovis canadensis*	...	...	40.0	2.30	17.39	Normal.
Big horn sheep	*Ovis canadensis*	F	...	68.2	4.42	15.43	Normal.
Tahr goat	*Hemitragus jemlaicus*	F	M	50.0	2.84	17.61	Pregnant.
Indian water buffalo	*Bubalus bubalis*	F	M	25.7	1.39	18.52	Normal.
Indian water buffalo	*Bubalus bubalis*	F	M	31.2	1.30	24.00	Normal.
Reindeer	*Rangifer tarandus*	F	6 mo.	30.3	2.23	13.59	Normal.
Thomson's gazelle	*Gazella thomsoni*	F	M	122.5	3.94	31.09	Normal.
Blesbok	*Damaliscus albifrons*	M	M	31.9	2.50	12.76	Normal.
Springbok	*Antidorcas euchore*	M	1 yr.	69.9	3.37	20.75	Normal.
Axis deer	*Cervus axis*	M	1 yr.	45.6	2.54	17.95	Normal.
White-tailed gnu	*Connochaetes gnu*	M	M	50.7	2.97	17.07	Normal.
Sika deer	*Cervus nippon*	M	2 mo.	102.6	3.70	27.73	Normal.
African burro	*Equus asinus*	F	M	30.5	2.78	10.97	Pregnant.
Indian elephant	*Elephas maximus*	F	19 mo.	123.1	3.09	39.84	Osteoporosis.
Brown bear	*Ursus sheldoni*	M	10 yr.	42.9	2.98	14.40	Normal.
Sitatunga	*Limnotragus spekei*	...	...	37.7	3.15	11.97	Normal.
Kenai wolverine	*Gulo luscus*, var. *katschenmakensis*	F	12 yr.	88.1	4.76	18.52	Normal.
Spotted hyena	*Crocuta crocuta*	M	4 yr.	15.5	1.64	9.45	Normal.
Wallaroo	*Macropus robustus*	M	...	10.7	1.20	8.92	Osteosarcoma of tibia.
Jaguar	*Panthera onca*	...	...	26.0	2.34	11.11	Normal.
Alligator	*Alligator mississippiensis*	M	M	155.9	5.16	30.21	Normal.
Sea lion	*Zalophus californianus*	M	20 yr.	78.4	4.98	15.74	Disseminated squamous cell carcinoma.

* M = Mature.

The ratio of protein to sialic acid was also increased above normal to 19.81 ± 4.63 in 7 cattle. Statistically, the seromucoid-protein, protein-bound sialic acid, and the ratio of protein to sialic acid were also significantly increased above normal in the diseased cattle ($p = < 0.01$).

Eight sheep with varying degrees of inflammation had plasma seromucoid levels of 86.3 ± 33.5 mg. of protein. Five of these sheep had protein-bound sialic acid levels of 4.79 ± 2.01, with elevated protein/sialic acid ratios of 18.2 ± 2.8. The differences between both seromucoid constituents (protein and sialic acid) in pathologic and normal sheep plasma were highly significant ($p = < 0.01$), while the increased ratio of protein to sialic acid in the sick sheep was also significantly elevated ($p = 0.05$).

Plasma seromucoid levels are also presented for various zoo species (table 6).

DISCUSSION

The statistically significant increases in plasma seromucoid levels of animals with various inflammatory processes suggest that any pathologic state with moderate to severe degeneration of the tissues may directly or indirectly elevate this glycoprotein fraction. The nonspecificity of the clinical condition responsible for the elevation in the plasma seromucoid level was quite apparent. The elevation of seromucoid levels in sheep and cattle appeared to parallel the severity of the inflammatory process of the various diseases studied. In the present study, ruminants with pathologic conditions and degrees of inflammation similar to those observed in horses were found to have significantly greater increases in their seromucoid fraction than those observed in horses. Of special interest was the finding that, in plasma samples obtained from both diseased domestic animals and specific exotic species (Thomson's gazelle, Indian elephant, sika deer, and alligator) with marked elevations of seromucoid, increases in sialic acid were proportionately smaller than increases in total protein.

Recent investigations have indicated that asymmetrically shaped, protein-bound hexose conjugates in the serum may be respon-

sible components concerned with an increased erythrocyte sedimentation rate (ESR). In tuberculosis and rheumatoid arthritis, a significant correlation between the total serum carbohydrate level and the ESR has been reported.[4,18] Due to the insignificant ESR (3–10 mm. in 24 hours) for both normal and diseased sheep and cattle,[15] the test is of little use clinically in these species. The plasma seromucoid level, however, could be used prognostically as a nonspecific index of the presence of inflammatory response or tissue destruction, or both, in sheep and cattle. Clinical conditions, such as tetanus in the horse, sheep, and goat (table 3, 5), which are not of an inflammatory nature, were not associated with elevated seromucoid levels.

Since the site of much of the total protein-bound hexose production is the liver, it has been observed that the plasma seromucoid level decreases in rabbits and man with parenchymatous liver disease.[18] It was of special interest in the present investigation that no drop in the seromucoid levels occurred in 2 horses (table 3) with subacute hepatic necrosis. One horse had bilirubinemia (7.8 mg./100 ml.), clinical icterus, and an increased bromsulfophthalein half-time of 8.0 minutes.[6] A horse with generalized plasma-cell myelomatosis and a serum *beta* ("myeloma") glycoglobulin of over 7 Gm./100 ml. of serum had a slightly elevated plasma seromucoid level in contrast to the reduced level frequently observed in this condition in man.[9]

SUMMARY

Plasma seromucoid levels were determined for normal horses, cattle, swine, sheep, and goats. This plasma glycoprotein fraction was expressed in terms of both its protein and protein-bound sialic acid content. A partial chemical analysis of the composition of the plasma seromucoid fraction in the horse, cow, pig, sheep, and goat was presented. Plasma from hospitalized animals with various inflammatory and degenerative changes was found to have increased seromucoid levels. Marked elevations of seromucoid occurred in the bovine and ovine plasma and appeared to parallel the degree of tissue inflammation or destruction, or both. Plasma seromucoid levels were also determined in various exotic animals caged at the San Diego Zoo.

References

[1] Boas, N. F., and Peterman, A. F.: Effects of Age, Food Intake, and Stress on Plasma Hexosamine Levels in the Rat. Proc. Soc. Exptl. Biol. & Med., 82, (1953): 19.

[2] Boyce, W. H., King, J. S., Jr., Little, J. M., and Artom, C.: Total Nondialyzable Solids (TNDS) in Human Urine. II. A Method for Reproducible Fractionation. J. Clin. Invest., 37, (1958): 1658.

[3] Bywater, H. W.: Über Seromucoid. Biochem. Ztschr., 15, (1909): 322.

[4] Cohen, R. J., and Byham, B.: Variations in the Serum Polysaccharide and Sedimentation Rate in Clinic Patients. J. Lab. & Clin. Med., 35, (1950): 869.

[5] Cornelius, C. E., Moulton, J. E., and McGowan, B., Jr.: Ruminant Urolithiasis. I. Preliminary Observations in Experimental Ovine Calculosis. Am. J. Vet. Res., 20, (Sept., 1959): 863–871.

[6] Cornelius, C. E., and Wheat, J. D.: Bromsulphalein Clearance in the Horse—A Quantitative Liver Function Test. Am. J. Vet. Res., 18, (April, 1957): 369–374.

[7] Elson, L. A., and Morgan, W. T. J.: A Colorimetric Method for the Determination of Glucosamine and Chondrosamine. Biochem. J., 27, (1933): 1824.

[8] Freund, E.: Über das Vorkommen von thierischem Gummi im normalen Blute. Zentralbl. Physiol., 6, (1893): 345.

[9] Greenspan, E. M.: Clinical Significance of Serum Mucoproteins. Advances Int. Med., 7, (1955): 101.

[10] Klenk, E., and Stoffel, W.: Über Neuraminsäurehaltige Glykoproteide des Blutserums. Ztschr. physiol. Chem., 302, (1955): 286.

[11] Kushner, D. S., Honig, K., Dubin, A., Dynievicg, H. A., Bronsky, D., de la Huerga, J., and Popper, H.: Studies of Serum Mucoprotein (Seromucoid). II. Physiologic Variations and Response to Stress. J. Lab. & Clin. Med., 47, (1956): 409.

[12] Martensson, E., Raal, A., and Svennerholm, L.: Sialic Acids in Blood Serum. Biochim. et biophys. acta, 30, (1958): 124.

[13] Mayer, K.: Über eine Mucoidähnliche Substanz aus Serum. Ztschr. physiol. Chem., 275, (1942): 16.

[14] Pedersen, K. O.: Ultracentrifugal and Electrophoretic Studies of Fetuin. J. Physiol. Colloid. Chem., 51, (1947): 164.

[15] Schalm, O. W.: A Syllabus on Veterinary Hematology. University of California, Davis, (1959): 27, 37.

[16] Schloss, B.: Colorimetric Determination of Glucosamine. Anal. Chem., 23, (1951): 1321.

[17] Shetlar, M. R., Bryan, R. S., Forter, J. V., Shetlar, C. L., and Everett, M. R.: Serum Polysaccharide Levels in Experimental Inflammation. Proc. Soc. Exptl. Biol. & Med., 72, (1949): 294.

[18] Starey, Z.: Mucoproteins in Clinical Chemistry. Clin. Chem., 3, (1957): 557.

[19] Tadati, F.: Valore della Frazione Mucoproteica del Siero di Sangue in Aloune Malattie del Cane. Arch. Vet. Ital., 9, (1958): 557.

[20] Tornblom, N.: Adrenals and Serum Polysaccharides. II. Action of DDD on the Serum Polysaccharides in Rabbit. Acta med. scandinav., 153, (1955): 149.

[21] Weimer, H. E., and Moshin, J. R.: Serum Glycoprotein Concentration in Experimental Tuberculosis of Guinea Pigs. Am. Rev. Tuberc., 68, (1952): 594.

[22] Weimer, H. E., and Winzler, R. J.: Comparative Study of Orosomucoid Preparations in Sera of Six Species of Mammals. Proc. Soc. Exptl. Biol. & Med., 90, (1955): 458.

[23] Winzler, R. J.: Determination of Serum Glycoproteins. In "Methods in Biochemical Analysis." Vol. II. Interscience Publishers, Inc., New York, (1955): 279–304.

[24] Winzler, R. J.: Advances in Cancer Research. vol. I. Academic Press, New York, (1953): 503.

[25] Winzler, R. J., and Burk, D.: Blood Protease and Cancer. J.. Nat. Cancer Inst., 4, (1944): 417.

[26] Winzler, R. J.: Glycoproteins of Plasma. In "Ciba Foundation on Chemistry and Biology of Mucopolysaccharides." Little, Brown and Co. (1958): 245.

[27] Wolfson, W. G., Cohn, C., Calvary, E., and Ichiba, F.: Studies in Serum Proteins: Rapid Procedure for Estimation of Total Protein, True Albumin, Total Globulin, *Alpha* Globulin, *Beta* Globulin, and *Gamma* Globulin in 1.0 ml. of Serum. Am. J. Clin. Path., 18, (1948): 723.

[28] Zanetti, C.: Über das Ovimucoid und über ein neues Glykoproteid aus dem Blutserum. Ann. chim. farm., 12, (1897): 1.

SUMMARIO IN INTERLINGUA

Nivellos de Seromucoide in Normal e Hospitalisate Animales de Species del Pais e Exotic

Le nivellos de seromucoide del plasma esseva determinate pro normal cavallos, bestial, porcos, oves, e capras. Iste fraction glycoproteinic del plasma esseva exprimite tanto per su contento de proteina como etiam per su contento de acido sialic ligate a proteina. Es presentate un partial analyse chimic del composition del fraction de seromucoide del plasma pro le cavallo, le vacca, le porco, le ove, e le capra. Esseva trovate que le plasma de hospitalisate animales con varie affectiones inflammatori e degeneratori se distingue per augmentate nivellos de seromucoide. Marcate elevationes de seromucoide occurreva in le plasmas bovin e ovin in parallela, apparentemente, al grado del inflammation e/o destruction tissular. Le nivellos de seromucoide in le plasma esseva etiam determinate in varie animales exotic al jardin zoologic de San Diego.

Survival on Pasture of Infective Larvae of *Cooperia pectinata* and *Ostertagia ostertagi*

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THE SURVIVAL TIME on pasture of the larvae of various species of gastrointestinal parasites has been determined by Kauzal [6] in Australia, Taylor [8] in England, and Dinsburg [2] at Beltsville, Md. They placed infective larvae on plots of grass free of other nematodes and recovered these larvae at various intervals by the use of the Baermann apparatus.

To develop a rotation program for grazing, it is necessary to know how long the larvae survive under the climatic conditions existing in any particular area.

MATERIALS AND METHODS

The infective larvae used in these trials were obtained by culturing feces from donor calves harboring infections of *Cooperia pectinata* or *Ostertagia ostertagi*, using the sphagnum moss method described by Cauthen.[1] The larvae were recovered from the cultures by the Baermann technique after 15 days to 25 days. The larvae were counted by dilution methods and placed on experimental plots of grass.

The grass plots were prepared as follows: An area of pasture without shade was fenced and all vegetation was removed. The plot was then covered and fumigated with methyl bromide. The area remained covered for five days. After the cover was removed, the area remained vacant for several days. The individual plots were formed by using galvanized metal grids, 6 inches square and 6 inches deep, placed in the soil to a depth of 4 inches, leaving 2 inches above the surface. Bermuda grass was planted in each square and over the entire test area. Subsequently, other plots were formed by placing 6-inch-diameter metal cylinders 8 inches deep in the soil with 2 inches left above surface. The grass was allowed to establish a firm turf before the trials began. The grass was kept clipped even with the top of the metal forms and clippings were left in the plot.

The larvae were washed out of the container with approximately 20 cc. of water and placed on the plots at the base of the grass.

When examining a plot for larvae, all vegetation and approximately 1 inch of topsoil were placed for 24 hours in a Baermann apparatus, lined with a double thickness of cheesecloth. The material that passed through the apparatus was then examined and larvae counted.

In all instances, material from individual squares or cylinders was examined 24 hours after the placing of larvae, and then at weekly intervals until no larvae were found. When a plot was examined and no larvae were found, three more plots were examined immediately to be sure that no larvae had survived.

RESULTS AND DISCUSSION

Many methods for determining the survival of parasitic nematode larvae have been reported.[2-8] The method used in this trial was selected to eliminate errors caused by variability in susceptibility of test animals.

The results of these trials, together with the daily records of maximum and minimum air temperature, humidity, and rainfall, are shown for *C. pectinata* (table 1) and for *O. ostertagi* (table 2).

It was found in some trials that larvae in some individual plots would survive longer than would comparable larvae in other plots of grass. To compensate for this, whenever no larvae were recovered from one plot other plots were examined immediately. In plots with a heavy cover of grass, more larvae were recovered than in plots with a sparse grass cover.

In all trials, the number of larvae recovered was very small after approximately 50 days. In the few plots where larvae survived over 100 days, very small numbers of larvae were recovered during the last five weeks. No larvae could be recovered from the majority of the plots after 90 days. There was little or no correlation between total length of survival, average maximum temperature, and relative humidity or rainfall.

SUMMARY

At College Station, Texas, the survival on pasture of infective larvae was determined for *Cooperia pectinata* in seven

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TABLE 1—Survival of Cooperia pectinata Infective Larvae on Pasture

Trial No.	Date		No. of larvae		Survival time (days)		Temperature				Relative humidity			Rainfall		
	Begun	Ended	Placed	Recovered (24 hr.)	50%	0%	Mean max.	Mean min.	Max.	Min.	Mean	Max.	Min.	Total inches	Days rain	Max. consec. days, no rain
1	3/13/57	4/30/57	10,000	2,890	35	54	72.6	53.7	83	35	86.5	100	68	17.82	30	4
2	10/20/57	3/27/58	5,000	3,780	46	138	63.1	44.1	86	22	86.4	100	71	21.51	60	21
5	8/14/58	10/20/58	5,000	3,600	10	68	85.4	68.3	100	54	85.6	100	73	12.15	35	9
6	8/14/58	10/11/58	9,000	7,200	12	59	87.7	69.6	100	54	85.9	100	72	12.09	31	21
7	8/19/58	10/30/58	5,000	3,730	11	71	85.2	69.2	98	50	89.1	100	72	14.49	37	10
10	10/17/58	1/ 6/59	5,000	1,840	22	81	65.5	44.4	86	15	86.1	100	71	7.05	26	9
12	4/ 6/59	8/10/59	5,000	2,600	30	118	92.8	73.3	99	37	87.9	100	62	23.19	31	17

TABLE 2—Survival of Ostertagia ostertagi Infective Larvae on Pasture

Trial No.	Date		No. of larvae		Survival time (days)		Temperature				Relative humidity			Rainfall		
	Begun	Ended	Placed	Recovered (24 hr.)	50%	0%	Mean max.	Mean min.	Max.	Min.	Mean	Max.	Min.	Total inches	Days rain	Max. consec. days, no rain
3	7/17/58	10/20/58	5,000	2,400	20	95	89.1	76.6	100	52	78.3	100	62	12.25	38	17
4	7/30/58	10/16/58	5,000	1,855	36	78	88.6	69.8	100	54	83.7	100	66	12.19	36	10
8	8/21/58	10/28/58	5,000	3,600	11	61	85.5	67.7	97	63	86.6	100	72	11.22	31	10
9	10/17/58	1/ 8/59	5,000	1,620	28	81	63.7	44.9	85	15	85.0	100	71	7.05	26	8
11	2/ 6/59	6/30/59	14,000	9,400	25	144	76.8	59.7	96	32	82.0	100	68	19.51	43	20

trials and for *Ostertagia ostertagi* in five trials.

The maximum length of survival was 138 days for *C. pectinata* and 144 days for *O. ostertagi*. The maximum time required for 50 per cent of the larvae to die was 46 days for *C. pectinata* and 36 days for *O. ostertagi*. The temperature, humidity, and rainfall during the period of the trials were recorded.

References

[1] Cauthen, G. E.: A Method of Culturing Large Numbers of *Haemonchus contortus* Larvae from Eggs in Feces. Proc. Helminthol. Soc. Washington, 7, (1940): 82.

[2] Dinaburg, A. G.: Development and Survival Under Outdoor Conditions of Egg and Larvae of the Common Ruminant Stomach Worm, *Haemonchus contortus*. J. Agric. Res., 69, (1944): 421–433.

[3] Doll, E. R., and Hull, R. E.: Survival of Nematode Parasites of Sheep on Pasture During Summer Months. Kentucky Agric. Exper. Station Bull. 482, (1946).

[4] Goldberg, A., and Rubin, R.: Survival on Pasture of Larvae of Gastrointestinal Nematodes of Cattle. Proc. Helminthol. Soc. Washington, 23, (1956): 65–68.

[5] Kates, K. C.: Overwinter Survival of Preparasitic Stages of Some Nematodes Parasitic in Sheep. Proc. Helminthol. Soc. Washington, 10, (1943): 23–25.

[6] Kauzal, G.: Examination of Grass and Soil to Determine the Population of Infective Larvae Nematodes on Pasture. Austral. Vet. J., 17, (1941): 181–184.

[7] Marquardt, W. C.: The Effect of Weather on the Development and Survival of the Free-Living Stages of *Nematodirus spathiger*. J. Parasitol., 45, (1959): 431.

[8] Taylor, E. L.: Observations on the Bionomics of Strongylid Larvae in Pastures. Vet. Rec., 50, (1938): 1265–1272.

SUMMARIO IN INTERLINGUA

Superviventia, in Pasturage, de Infective Larvas de *Cooperia pectinata* e *Ostertagia ostertagi*

A College Station in Texas, le superviventia in pasturage esseva determinate pro larvas de *Cooperia pectinata* in septe essayos e pro larvas de *Ostertagia ostertagi* in cinque.

Le duration maximal del superviventia esseva 138 dies pro *C. pectinata* e 144 dies pro *O. ostertagi*. Le tempore maximal requirite usque un medietate del larvas habeva morite esseva 46 dies pro *C. pectinata* e 36 dies pro *O. ostertagi*. Le temperatura, humiditate, e le precipitation durante le periodo del essayos esseva registrate.

Persistence of Foot-and-Mouth Disease Virus in Bovine Kidneys and Blood as Related to the Occurrence of Antibodies

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PRIMARY CULTURES of bovine and porcine kidney cells have been used extensively for study of foot-and-mouth disease virus (FMDV).[1,3,7] It was of interest to determine if cultures prepared from kidneys of animals previously infected with FMDV could be used to propagate the virus. In a preliminary experiment, it was found that FMDV persisted in calf kidneys for at least six days after intradermal inoculation of virus in the tongue. This persistence was evidenced by cytopathic changes referable to FMDV in many cell cultures of kidneys taken from animals on the first through the sixth postinoculation day. Cultures derived from the same kidneys, but not showing cytopathic changes, and cultures from kidneys of animals infected 14, 21, 28, and 72 days earlier were about as susceptible to infection with FMDV as cultures of tissue from unexposed animals.

The experiments described here confirmed the preliminary findings. Viremia, appearance of antibodies, and persistence of virus in the kidneys were studied. Attempts were also made to determine if virus in the kidneys was situated intracellularly.

MATERIALS AND METHODS

Animal Inoculations.—Eight 6-month-old calves were used. Blood samples were drawn and each calf was inoculated intradermolingually (i.d.l.) with 2 ml. of a 10 per cent suspension of bovine tongue tissue infected with FMDV, type A, strain 119 (FMDV-A119). Animals were killed one, two, four, six, 14, 21, and 28 days after inoculation. The eighth calf was given two additional inoculations of virus suspension, consisting of 2 ml. intravenously on the 36th day and 5 ml. intravenously on the 60th day. The animal was killed 72 days after initial injection, and its blood and kidneys were removed for testing.

Detection of Virus in Serum and Kidneys.—The serum was recovered as soon as possible after death. It was then serially diluted in Hanks' balanced salt solution containing 0.5 per cent lactalbumin hydrolysate and 2 per cent bovine serum (LC fluid). This mixture was assayed for virus content in cell cultures of kidneys from susceptible animals.

To detect virus in the kidneys, 1 Gm. of cortical tissue was ground in a mortar with an abrasive * and suspended in 9 ml. of LC fluid. After the suspension was cleared by centrifugation at 1,000 r.p.m. for 10 minutes, it was assayed for virus in bovine-kidney cell cultures. The remaining cortical tissue was dispersed by trypsin according to Youngner's modification[10] of the method of Dulbecco and Vogt.[5] The cells were washed three times by sedimentation in phosphate-buffered saline solution (PBS),[5] at 600 r.p.m. for five minutes. A portion of cells was ground in a mortar with the abrasive and mixed with nine parts LC fluid. The suspension was clarified by centrifugation at 1,000 r.p.m. for 10 minutes and inoculated into tube cultures of bovine kidney cells.

Culture of Kidney Cells from Convalescent Animals.—Other portions of the washed, trypsin-dispersed cells were suspended (1:200) in LC fluid and in LC fluid containing 2 per cent bovine FMDV-A119 immune serum. Suspensions were dispensed in 0.4-ml. amounts in culture tubes and incubated in a slanted position at 37 C. Sixty-four cultures were made in each type of fluid. They were examined daily under a microscope and fluids were changed every three days. Cultures with changes indicative of FMDV infection were stored at −30 C. until tested for virus by injection of steers.

Sensitivity of Cultured Cells to Infection.—On the eighth day of incubation, 30 cultures from kidneys of infected or recovered animals, growing in LC fluid and having no cytopathogenic effect, were used for assay of FMDV-A119. Moreover, forty of the cultures grown in the presence of immune serum were washed twice with 3-ml. amounts of LC fluid. After 0.4 ml. of medium was placed on each culture, they were returned to the incubator. Two days later, 30 of these cultures were used in a virus assay. The ten remaining cultures were examined daily during an additional 14 days for appearance of FMDV infection.

A single virus pool, the 65th tissue culture (TC)

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* Alundum, produced by the Norton Co., Worcester, Mass.

passage of FMDV-A119, was used in all titrations. It was stored at −30 C. in flame-sealed ampules. An ampule was thawed on each day that virus was required.

Each virus assay in cultures derived from kidneys of infected or recovered animals was accompanied by a titration of the same virus sample in bovine kidney cultures prepared from susceptible animals. Five cultures were inoculated with each decimal dilution of virus. Virus titer, expressed as t.c.i.d.$_{50}$, was calculated by the method of Reed and Muench.[8]

Serum Neutralizing Antibodies.—Serums prepared from blood samples taken before virus inoculation and at the death of each animal were assayed for neutralizing antibodies. Serial ten-fold dilutions of serums were mixed with equal amounts of a $10^{-4.5}$ dilution of the aforementioned TC virus pool. The mixtures were incubated one hour at 37 C. and assayed in tube cultures of bovine kidney cells. In titration of the virus pool, the dilutions were also held one hour at 37 C. before inoculation into cultures.

Possible Intracellular Presence of Virus in Kidneys.—Tube cultures were prepared of trypsin-dispersed kidney cells of animals killed five and six days after inoculation with FMDV-A119. Some of the cultures were grown in LC fluid and some in LC fluid containing bovine FMDV-A119 immune serum.

Other lots of dispersed cells were treated to eliminate or inactivate any virus that might have been on the cell surfaces or free in the suspension. After three washings in PBS, one lot of cells was suspended in Hanks' balanced-salt solution at pH 5.5 and held for ten minutes. The cells were recovered by centrifugation, and one portion was cultured in LC fluid and another in LC fluid containing FMDV-A119 antiserum. Still another lot of dispersed cells was washed three times in PBS containing 5 per cent bovine FMDV-A119 immune serum followed by a fourth washing with PBS without serum. Cells given this treatment were allotted to three portions. The first portion was cultured in LC fluid and the second in LC fluid containing antiserum. The third portion was exposed to pH 5.5 for ten minutes in Hanks' balanced-salt solution before being cultured.

RESULTS

Persistence of Virus in the Kidneys.—Virus was detected (table 1) in bovine kidney cell cultures inoculated with extracts of tissue or trypsin-dispersed cells from the kidneys of animals infected one, two, four, and six days previously. Infection also occurred in many of the cultures prepared from kidney cells from this same group of convalescent animals. In the latter instance, the cytopathic changes appeared during the fifth and sixth days of incubation and involved 16 to 66 per cent of the cultures prepared. Fluids from the infected cultures produced typical lesions of foot-and-mouth disease (FMD) when injected i.d.l. into steers, and materials from these lesions fixed complement in the presence of FMDV-A119 immune guinea pig serum. At no time was virus demonstrable in cells grown in the presence of immune serum.

The highest virus level in the kidneys was reached on the second day (fig. 1);

TABLE 1—Foot-and-Mouth Disease Virus Persistence in Calf Kidneys

Post-inoculation day	Virus titer (log i.d.$_{50}$/ml.)*		Spontaneous infection in cells cultured in normal serum**		
	Tissue	Cells	Incubation time (days)	Cultures infected** (No.)	(%)
1	3.03	1.90	6	11/64†	17
2	4.90	2.23	5	42/64	66
4	2.23	1.23	6	25/64	39
6	1.23	1.23	5	10/64	16
14	none detected	none detected	14	0/64	0
21	none detected	none detected	14	0/64	0
28	none detected	none detected	14	0/64	0
72‡	none detected	none detected	14	0/64	0

* Extracts and cell cultures were prepared from the same kidneys; extracts were assayed in kidney cell cultures from normal calves.

** Virus was not demonstrated at any time in cells grown in the presence of immune serum.

† Numerator = number of cultures positive; denominator = number of cultures used.

‡ Hyperimmunized, before killing, with two subsequent intravenous inoculations of virus (*see* Materials and Methods).

virus was recovered from tissue cultures and extracts through the sixth day but was no longer detected on the fourteenth day.

Occurrence of Neutralizing Antibodies in the Serum.—Virus was present in serum on the first and second days after inoculation. Neutralizing antibodies first became detectable on the fourth day and increased to a maximum by the fourteenth day. Progressive decreases in virus-neutralizing capacity of the serum occurred thereafter. The course of viremia and the persistence of virus in the kidneys, as compared to the appearance of antibodies in the blood is shown (fig. 1).

TABLE 2—Sensitivity of Cell Cultures to Foot-and-Mouth Disease Virus Infection

Post-inoculation day	Titers (log i.d.$_{50}$/ml.) of a single virus pool assayed in cell cultures* grown in LC fluid containing:			
	Normal serum		Immune serum	
	Test	Control	Test	Control
1	6.23	6.78	6.78	6.78
2	6.23	6.90	6.57	6.78
4	6.57	6.90	6.78	6.57
6	6.57	7.04	7.04	7.04
14	6.78	6.23	6.78	6.90
21	6.57	6.78	6.23	6.90
28	6.23	6.23	6.57	6.23
72**	6.78	6.57	6.90	6.57

* Test cultures were from kidneys of infected and recovered animals; control cultures were from kidneys of normal animals.

** Hyperimmunized before killing with two subsequent intravenous inoculations of virus.

Sensitivity of Cell Cultures to Foot-and-Mouth Disease Virus Infection.—The titers are shown (table 2) for duplicate samples of a single tissue culture passage of FMDV-A119, assayed in bovine kidney cultures prepared from uninoculated animals (*i.e.*, control cultures) and in cultures derived from kidneys of infected and recovered animals. Cultures prepared from kidneys of animals infected one, two, four, and six days earlier, which did not develop cytopathic changes in the presence of LC fluid, appeared to be slightly less sensitive to infection than control cultures. The differences were small but consistent. Cultures grown in the presence of immune serum and those derived from animals infected 14, 21, 28, and 72 days previously had about the same degree of susceptibility to infection as control cultures.

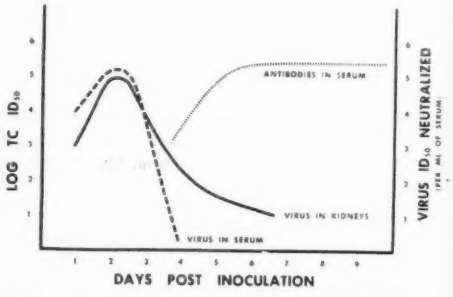

Fig. 1—Virus in bovine serum and kidneys in relation to appearance of serum neutralizing antibodies.

Location of Virus in the Kidneys.—Kidney tissue, taken from animals five and six days after i.d.l. inoculation, dispersed by tryptic action, and treated with immune serum and acidification (singly or in combination) to eliminate or inactivate extracellular virus, continued to give rise to significant numbers of infected cultures when grown in LC fluid (table 3). As before, infection was not encountered in cultures grown in fluids containing immune serum.

TABLE 3—Persistence of Infection in Cultures Prepared from Cells Treated to Remove Extracellular Virus

Days after inoc.	No treatment (regular culture preparation)			10 min. at pH 5.5			Washed 3 times with immune serum			10 min. at pH 5.5 and washed 3 times in immune serum		
	culture day	Infected		culture day	Infected		culture day	Infected		culture day	Infected	
		total	%		total	%		total	%		total	%
5	6th	23/64	36	8th	13/64	20	8th	15/64	23	12th	9/64	14
6	6th	11/64	17	8th	4/64	6	8th	5/64	8	13th	3/64	5

The percentages of infected cultures were smaller in the treated than in the untreated cultures; however, cultures of treated cells developed slowly and had fewer centers of cellular outgrowth. The greatest effect was

in cultures of cells given both treatments. Evidently, the treated-cell suspensions contained fewer viable cells.

DISCUSSION

Bovine kidney cell cultures derived from animals infected with or recovered from FMDV-A119 infection had no special resistance to that virus; therefore, such cultures may be used in production of this virus type. However, the cultures are not satisfactory for virus assay or for production of any other type of FMDV because of possible carry-over of infection from the tissue donor.

Although these practical considerations are important, certain basic implications of the experiments are of greater interest. Virus found in kidneys on the first and second days after i.d.l. injection may have been confined to the organ's blood supply, since virus was recovered from the serum on those days. On the fourth, fifth, and sixth days, however, virus present in the kidneys may have been present intracellularly or extracellularly at sites not easily reached by immune globulins, since neutralizing antibodies rather than virus were found in the serum on those days.

Additional evidence strongly supported the conclusion that FMDV persisted intracellularly in the kidneys of cattle for at least six days after i.d.l. injection. Extracellular virus may have been eliminated by rigorous washing of the cells during the dispersal process followed by additional washings in immune serum and, finally, exposure to hydrogen ion concentration. The latter procedure ordinarily produces rapid inactivation of FMDV.[2] However, a substantial number of the cultures prepared from cells subjected to such treatments underwent cytopathic changes referable to FMDV infection. Perhaps the virus responsible for these changes escaped elimination or destruction because it resided within intact cells.

Waldman *et al.*[9] reported the isolation of FMDV from urine of cattle several months after the animals had recovered from the infection. Recently, Van Bekkum *et al.*[8] succeeded in isolating FMDV from bovine saliva for several months following infection. Regardless of the bearing these findings may have on the importance of the carrier state of FMD, we need to learn how the virus is propagated and disseminated by animals possessing specific antibodies.

Van Bekkum and associates[8] suggest that virus recovered from saliva may be produced in cells lining the oral cavity. Here, as in the kidney, no pathologic changes have been reported to indicate any foci of virus formation. Perhaps these tissues should be more carefully examined. The affected areas may be small or the cells may be altered only slightly. If there are no visible changes in the cells, those harboring virus might be detected by fluorescence microscopy.[4]

Our work is not conclusive for establishing a carrier state; however, it does present a means of regularly producing a condition of coexistence of virus and its specific antibody in cattle and may serve as a convenient model to study the mechanism involved.

SUMMARY

In calves that had been experimentally infected with foot-and mouth disease virus, viremia existed during the first two days postinoculation. By the fourth day, detectable amounts of neutralizing antibodies were present; virus was no longer found in the blood.

Virus persisted in the kidneys through at least the sixth day and there was evidence that this virus might be located intracellularly.

References

[1] Bachrach, H. L., Hess, W. R., and Callis, J. J.: Foot-and-Mouth Disease Virus: Its Growth and Cytopathogenicity in Tissue Culture. Science, 122, (1955): 1269–1270.

[2] Bachrach, H. L., Breese, S. S. Jr., Callis, J. J., Hess, W. R., and Patty, R. E.: Inactivation of Foot-and-Mouth Disease Virus by pH and Temperature Changes and by Formaldehyde. Proc. Soc. Exptl. Biol. & Med., 95, (1957): 147–152.

[3] Bachrach, H. L., Callis, J. J., Hess, W. R., and Patty, R. E.: A Plaque Assay for Foot-and-Mouth Disease Virus and Kinetics of Virus Reproduction. Virology, 4, (1957): 224–236.

[4] Coons, A. H., and Kaplan, M. H.: Localization of Antigen in Tissue Cells. II. Improvements in a Method for the Detection of Antigen by Means of Fluorescent Antibody. J. Exptl. Med., 91, (1950): 1–13.

[5] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., 99, (1954): 167–182.

[6] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

[7] Sellers, R. F.: Growth and Titration of the Viruses of Foot-and-Mouth Disease and Vesicular Stomatitis in Kidney Monolayer Tissue Cultures. Nature, 176, (1955): 547–549.

[8] Van Bekkum, J. G., Frenkel, H. S., Frederiks, H. H. J., and Frenkel, S.: Observations on the Carrier State of Cattle Exposed to Foot-and-Mouth Disease Virus. Tijdschr. Diergeneesk., 20, (1959): 1159–1164.

[9] Waldmann, O., Trautwein, K., and Pyl, G.: Die Persistenz des Maul-und Klauenseuchevirus im Körper durchgeseuchter Tiere und Seine Ausscheidung. Zentralbl. Bakt., 121, (1931): 19–32.

[10] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., 85, (1954): 202–205.

SUMMARIO IN INTERLINGUA

Le Persistentia de Virus de Febre Aphthose in Bovin Ren e Sanguine in Relation al Occurrentia de Anticorpore

In vitellos, experimentalmente inficite con virus de febre aphthose, viremia existeva durante le prime duo dies post le inoculation. Le quarte die, detegibile quantitates de anticorpore neutralisante esseva presente, e nulle virus esseva incontrate in le sanguine.

In le renes, virus persisteva durante al minus sex dies. Le evidentia pareva indicar que iste virus esseva locate intra le cellulas.

The Effect of Growth Factors on the Growth of *Vibrio fetus*

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THE RATHER SLOW growth of *Vibrio fetus* in conventional mediums has presented a considerable problem to every worker in the field of bovine vibriosis. Inherent slow rate of growth or failure to provide the organism with suitable artificial environment might be assumed to be the chief reason for this phenomenon. The nutritional requirements of bovine strains of *V. fetus* have not been studied in detail. The indirect and limited knowledge in this field is derived from studies where several mediums have been compared for ability to support the growth of the organism. All these mediums are characterized by complex composition. Practically all contain biological ingredients of uncertain composition. This situation also exists in the case of the Brucella broth (Albimi) medium.

The authors showed in previous studies [39] that this medium with 0.1 per cent agar added provided the most favorable environment for growth of the strains of *V. fetus* employed. The principal constituents of the medium are peptone "M" and yeast autolysate. Similar preparations are known to contain bacterial nutrients such as amino acids and vitamins. The lack of knowledge of the exact composition of this medium suggested that an enrichment of it by addition of certain growth factors might result in an earlier and more copious growth of *V. fetus*.

This study was designed to investigate the effect of a number of additives, including reducing substances, metals, products of glycolytic metabolism, purines, pyrimidines, and some miscellaneous growth factors.

MATERIALS AND METHODS

Cultures.—Of the 22 catalase-positive bovine strains of *V. fetus* employed in this study, 15 strains had been isolated from aborted fetuses and seven from semen.

Basal Medium.—Brucella broth (Albimi), containing 0.1 per cent agar, served as the basal medium in this experiment.

Growth Factors.—Twenty-one substances, including metals, metabolites, and miscellaneous growth factors, were selected for use in this experiment.

Methods.—Additional observations at six and 12 hours following inoculation represent the only deviation from the experimental methods described in a previous publication.[39] These two additional observation periods were incorporated to detect the first visible growth of *V. fetus*.

RESULTS

The effects of the additives were found to vary from stimulation to inhibition of *V. fetus*, depending upon the substance and concentration employed. The results are summarized (table 1).

DISCUSSION

Since the basal medium is not chemically defined, the results of this investigation can not be interpreted as a true reflection of the nutritional requirements of the bovine strains of *V. fetus*. An evaluation of the results is limited to speculation for the same reason.

Thiols, such as thioglycollate and glutathione, have been reported to enhance the growth of *V. fetus*.[11-13] Glutathione* did not affect the organism in this study. Cysteine,** considered to be the biologically active constituent of glutathione, produced

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* Glutathione, reduced monosodium, supplied by Schwarz Laboratories Inc., Mount Vernon, N.Y.

** L-cysteine hydrochloride, supplied by California Foundation for Biochemical Research, Los Angeles, Calif.

TABLE 1—The Effect of Various Levels of Growth Factors on the Growth of Vibrio fetus

Growth factor	Concentration (%)		
	Stimulatory	Inhibitory	Without effect
Glutathione	.....	.....	0.00625–0.5
Cysteine	0.002–0.03	0.25–1.0	.....
Mg	0.01	.....	.....
Mn	0.0025–0.005	.....	0.01–0.02
Fe	0.015–0.03	0.125	0.06*
Mo	0.015–0.03*	0.03–0.06**	0.06*
Co	.....	0.125–1.0†	0.005–0.025
Pyruvate	.....	0.05–1.0	0.015–0.5
Lactate	0.015–0.5	.....	1.0
Oxalacetate	.....	.....	0.01–0.15
Fumarate	.....	0.015–1.0	.....
α-Ketoglutarate	0.05	0.1–0.2	.....
Glutamate	0.1–0.25	.....	.....
Glutamine	0.1–0.5	.....	.....
Aspartate	.....	.....	0.006–0.1
Uracil	0.1	.....	.....
Thymine	0.1	.....	.....
Adenine	.....	0.1–0.2	0.05
Urea	.....	0.6–1.0	0.1–0.5
PABA	0.015–0.025	0.1–0.5	0.05
17-β-Estradiol	0.005	0.01–0.2	.....

* Aerobic gaseous environment. ** Under 15 per cent CO_2 tension. † Inhibition regardless of the gaseous environment.

marked stimulation of the *V. fetus* strains studied. The different responses are difficult to explain. Differences in the biological activity of both thiols have been demonstrated.[7,30] This would suggest that *V. fetus* also utilizes cysteine for functions other than that of a precursor to glutathione and participating in reduction reactions. The inhibition obtained by higher levels of this amino acid appears to indicate that it is involved in more than one metabolic reaction.

Magnesium has been shown to stimulate carboxylase of yeast.[17] The metal has also been shown to stimulate the growth of several bacteria.[2,19,25,38] All levels of magnesium chloride* employed in this study produced enhanced growth of *V. fetus*. The greatest increase in growth rate was observed in mediums containing 0.1 per cent of magnesium chloride.

Manganese is another metal that has received attention as a growth factor for bacteria.[8,25,27,29] The *V. fetus* strains used in this study had definite increases in growth in the presence of 0.0025 and 0.005 per cent of manganese chloride** in the medium. Marked visible growth was apparent as early as 12 hours following inoculation.

Iron (ferrous sulfate**), required by certain bacteria for optimal growth,[9,27,35,38] caused only slight stimulation of *V. fetus* when used in concentrations not exceeding 0.03 per cent. The inhibition produced by the higher levels of iron was markedly augmented by incubation in a 15 per cent carbon dioxide atmosphere, indicating that a more rapid utilization of the metal occurs in normal atmospheric air.

Molybdenum is one of the trace elements considered to be essential for animals, plants, and bacteria. The actual mode of action is, however, not understood.[18,21] The effects of molybdenum trioxide** in this study were strikingly dependent upon the carbon dioxide concentration in the gaseous environment. The same concentration of the metal that caused slight, but definite, stimulation of *V. fetus* incubated aerobically inhibited the growth of the organism in an environment containing 15 per cent carbon dioxide.

The only metal used in this study without a stimulatory effect on *V. fetus* was cobalt acetate.* Levels below 0.05 per cent did not affect the growth of the organism. Higher concentrations of the metal were bactericidal to *V. fetus*. Similar adverse effect of cobalt upon other bacteria has been reported.[1,28,29]

Enzyme systems participating in the various metabolic reactions involving pyruvate and lactate have been demonstrated in several bacteria.[6,37] In this study, py-

* Merck Reagent, Merck and Company, Inc., Rahway, N.J.

** Analytical Reagent, Mallinkrodt Chemical Works.

ruvate and lactate were incorporated in the test mediums in identical concentrations. The effect of these substances was, however, found to be strikingly different; while the 0.15 to 0.5 per cent levels of pyruvate did not affect the growth of *V. fetus*, similar concentrations of lactate produced marked stimulation of growth. Lactate concentrations as high as 1.0 per cent did not inhibit the growth of *V. fetus*. The same level of pyruvate caused complete inhibition of the organism. Alexander [4] reported that 0.2 per cent of either of these acids enhanced the growth of some strains of *V. fetus* in a defined medium. The discrepancy between his findings and the observations of this study might be a result of differences existing between the ovine and bovine strains in regard to their ability to utilize pyruvate. The possibility that the different response to the added pyruvate might be due to the different compositions of the basal mediums cannot be entirely eliminated. Oxalacetate has been shown to serve as a substitute for carbon dioxide for growth of bacteria requiring this gas as an essential growth factor.[36] The *V. fetus* strains employed in this experiment were not affected by the levels of oxalacetic acid* employed. Alexander [4] reported similar observations.

Fumarate represents one of the members of the Krebs' cycle linking carbohydrate metabolism with that of amino acids. The ammoniation reaction has been described in many bacteria.[23] According to Alexander,[4] fumarate is utilized by ovine strains of *V. fetus*. The findings of the present study disagree with his observations, since all levels of fumaric acid (purified)* caused inhibition of the bovine strains studied. This disagreement might reflect metabolic differences between the ovine and the bovine strains of *V. fetus*. Utilization of aspartate has been demonstrated in several bacteria.[16, 25, 32] The concentrations of l-aspartic acid** used in this study did not affect the growth of *V. fetus*. Similar observations have been made by Alexander,[4] who studied ovine strains of the organism.

According to Alexander,[4] α-ketoglutarate enhanced the growth of ovine strains of *V. fetus*. In the present study, the effect of α-ketoglutaric acid** was found to depend upon the level employed. Thus, marked stimulation was observed in the presence of 0.05 per cent of α-ketoglutarate. The higher levels, however, caused complete inhibition of the organism.

Glutamate and its amine, glutamine, are common constituents of many mediums.[5, 14, 16, 22, 32] Alexander,[4] who employed l-glutamate, but not glutamine, concluded that ovine strains of *V. fetus* are unable to utilize the former growth factor. The observations of this study indicate that the bovine strains of *V. fetus* are capable of utilizing both l-glutamate monosodium fied)* and l-glutamine* interchangeably.

Several bacteria, including certain gram-negative organisms, have been shown to require uracil for optimal growth.[14, 20, 24] It has also been reported that uracil serves as a precursor for thymine synthesis. This might explain the similar stimulatory effect produced by both pyrimidines** in this study.

According to available reports,[20, 22] *Brucella abortus* and certain other bacteria utilize adenine (l-aspartic acid).* The *V. fetus* strains used in this experiment appeared to be unable to utilize this purine. In fact, levels of 0.1 per cent and higher produced inhibition. Urea is considered to serve as the nitrogen donor for bacteria capable of hydrolyzing this substance.[22, 27] Rolle and Mundt [26] reported selective stimulation of *V. fetus* by urea. Similar concentrations of urea† used in the present study failed to stimulate the organism. Kuzdas and Morse [15] showed that *V. fetus* does not possess the enzyme urease, thus providing explanation for the observations in this experiment.

P-aminobenzoic acid (PABA) has received much attention since Fildes [10] demonstrated that the growth factor counteracts the bacteriostatic effect of sulfanilamide. The observations of this study appear to indicate that *V. fetus* has only a limited ability to utilize PABA.†

According to certain authors,[3, 33] the genital tract of the cow is most favorable for *V. fetus* during the extrogenic phase of

* Supplied by California Foundation for Biochemical Research, Los Angeles, Calif.

** Supplied by Nutritional Biochemical Corp., Cleveland, Ohio.

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** Supplied by California Foundation for Biochemical Research, Los Angeles, Calif.

† Supplied by Merck and Co., Inc., Rahway, N.J.

the estrous cycle. 17-β-estradiol is known to stimulate certain metabolic reactions.[34] Stimulation of growth of *V. fetus* was observed in this study in presence of 0.005 per cent of 17-β-estradiol.* This stimulatory effect was most apparent in the cultures incubated aerobically. This observation suggests that incorporation of the hormone in mediums employed in diagnostic work might favor isolation of *V. fetus* without incubation in complex gaseous environment.

SUMMARY AND CONCLUSIONS

1) The effect of 21 growth factors on the growth of *Vibrio fetus* was studied in this experiment.

2) Certain concentrations of cysteine, magnesium, manganese, iron, lactate, α-ketoglutarate, glutamate and glutamine, uracil, thymine, p-aminobenzoic acid, and 17-β-estradiol enhanced the growth of *V. fetus* when added to the basal medium.

3) The results indicate that the slow growth rate of *V. fetus* in conventional mediums is partially due to certain deficiencies of the mediums.

* Supplied by the Sigma Chemical Co., St. Louis, Mo.

References

[1] Abelson, P. H., and Aldous, E.: Ion Antagonisms in Microorganisms: Interference of Normal Magnesium Metabolism by Nickel, Cobalt, Cadmium, Zinc, and Manganese. J. Bact., 60, (1950): 401–413.

[2] Ackart, B. W., and Murray, T. J.: A Medium for the Rapid Cultivation of *Mycobacterium avium*. J. Bact., 62, (1951): 75–79.

[3] Adler, H. C.: Diagnosis of *Vibrio fetus* Carrier Bulls by Intravaginal Inoculation of Guinea Pigs. Nord. Vet.-med., 5, (1952): 454–459.

[4] Alexander, J. D.: The Utilization of Carbohydrate Intermediates and Amino Acids by *Vibrio fetus*. M.S. thesis, Montana State College, Bozeman, 1954.

[5] Ayengar, P., Roberts, E., and Ramasarma, G. C.: Roles of Glutamine in Growth of *Lactobacillus arabinosus*. J. Biol. Chem., 193, (1951): 781–791.

[6] Barron, E. S. G., and Hastings, A. B.: Studies on Biological Oxidation. II. The Oxidation of Lactic Acid by Hydroxyoxidase and Its Mechanism. J. Biol. Chem., 100, (1933): 155–181.

[7] Behrens, O. K.: Co-Enzymes for Glyoxalase. J. Biol. Chem., 141, (1941): 503–508.

[8] Cole, L. J.: Suppression of Colonial Variation in *Brucella abortus* by Metal-Coupling Agents. J. Bact., 64, (1952): 847–853.

[9] Elvehjem, C. A.: The Role of Iron and Copper in the Growth and Metabolism of Yeast. J. Biol. Chem., 90, (1931): 111–132.

[10] Fildes, P.: The Mechanism of the Antibacterial Action of Mercury. Brit. J. Exptl. Path., 21, (1940): 67–73.

[11] Hansen, P. A., Price, K. E., and Clements, M. F.: Suitable Thioglycolate Media for the Cultivation of *Vibrio fetus*. J. Bact., 64, (1952): 772.

[12] Huddleson, I. F.: A Satisfactory Medium for the Isolation, Cultivation and Maintenance of Viability of *Vibrio fetus*. J. Bact., 56, (1948): 508.

[13] Hughes, D. E., and Gilman, H. L.: A Satisfactory Method for the Culture of *Vibrio fetus*. Cornell Vet., 63, (1953): 563–565.

[14] Knight, B. C. J. G.: Growth Factors in Microbiology. Vitamins and Hormones, 3, (1945): 108–277.

[15] Kuzdas, C. D., and Morse, E. V.: A Selective Medium for the Isolation of *Vibrio fetus* and Related Vibrios. J. Bact., 77, (1956): 251–252.

[16] Lichstein, H. C., and Cohen, P. P.: Transamination in Bacteria. J. Biol. Chem., 157, (1945): 85–91.

[17] Lohman, K., and Schuster, P.: Untersuchungen über die Cocarboxylase. Biochem. Ztschr., 292, (1937): 188–214.

[18] Mahler, D. K., Mackler, B., and Green, D. E.: Studies on Metalloflavoproteins. III. Aldehyde Oxidase, a Molybdoprotein. J. Biol. Chem., (1954): 210–465.

[19] Myers, J.: Studies on the Spirillae. Methods of Isolation and Identification. J. Bact., 40, (1940): 705–711.

[20] Newton, J. W., and Wilson, J. B.: CO_2 Requirements and Nucleic Acid Synthesis by *Brucella abortus*. J. Bact., 68, (1954): 74–76.

[21] Nicholas, B. J. D., Nason, A., and McElroy, W. D.: Molybdenum and Nitrate Reductase. J. Biol. Chem., 207, (1954): 341–351.

[22] Porter, J. R.: Bacterial Chemistry and Physiology. John Wiley & Sons, Inc., New York, 1946.

[23] Quastel, J. H., and Woolf, B.: The Equilibrium Between 1-Aspartic Acid, Fumaric Acid and Ammonia in the Presence of Resting Bacteria. Biochem. J., 20, (1926): 545–555.

[24] Richardson, M. G.: The Nutrition of *Staphylococcus aureus*, Necessity for Uracil in Anaerobic Growth. Biochem. J., 30, (1936): 2134–2190.

[25] Rode, L. J., Oglesby, C., and Schuhardt, V. T.: The Cultivation of Brucellae in Chemically Defined Media. J. Bact., 60 (1950): 661–668.

[26] Rolle, M., and Mundt, W.: Die Züchtung von *Vibrio fetus*. Spirillum Suis und ähnlichen Microorganismen. Zentralbl. Vet.-med., 11, (1955): 451–458.

[27] Sanders, T. K., Higuchi, K., and Brewer, C. D.: Studies on the Nutrition of *Brucella melitensis*. J. Bact., 66, (1953): 294–299.

[28] Schade, A. L.: Cobalt and Bacterial Growth with Special Reference to *Proteus vulgaris*. J. Bact., 56, (1949): 811–822.

[29] Shankard, K., and Bard, R. C.: The Effect of Metallic Ions on the Growth and Morphology of *Clostridium perfringens*. J. Bact., 63, (1952): 279–289.

[30] Slater, E. C.: A Respiratory Catalyst Required for the Reduction of Cytochrome c by Cytochrome b. Biochem. J., 45, (1949): 14–30.

[31] Snell, E. E.: Bacterial Nutrition—Chemical Factors. In "Bacterial Physiology." Academic Press, Inc., New York, (1951): 215–255.

[32] Stumpf, P. K., and Green, D. E.: 1-Amino Acid Oxidase of *Proteus vulgaris*. J. Biol. Chem., 153, (1944): 387–399.

[33] Vandeplassche, M., Paredis, F., Van Nievenhuyse, E., Brone, E., and Florent, A.: Beiträge zur Vibrio-Foetus-Infektion bei Rindern mit besonderer Berücksichtung der Unfruchtbarkeit. Zentralbl. Vet.-med., 3, (1956): 111–130.

[34] Villie, C. A., and Gordon, E. E.: The Stimulation of a DPN-Linked Isocitric Dehydrogenase from Human Placenta. Bull. Soc. Chem. Belg., 65, (1956): 186–201.

[35] Waring, W. S., and Werkman, C. H.: Iron Requirements of Heterotrophic Bacteria. Arch. Biochem., 1, (1943): 425–433.

[36] Werkman, C. H.: Assimilation of Carbon Dioxide by Heterotrophic Bacteria. Bacterial Physiology, Academic Press, Inc., New York, (1951): 404–428.

[37] Werkman, C. H., and Schlenk, F.: Anaerobic Dissimilation of Carbohydrates. In "Bacteria Physiology." Academic Press, Inc., New York, (1951): 282–324.

[38] Young, E. C., Begg, R. W., and Pentz, E. I.: The

Inorganic Nutrient Requirements of *Escherichia coli*. Arch. Biochem., 6, (1945): 121–136.

[39] Zemjanis, R., and Hoyt, H. H.: The Effect of Enzyme Inhibitors on *Vibrio fetus*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. Am. J. Vet. Res., 21, (Nov., 1960): 1109–1113.

SUMMARIO IN INTERLINGUA

Le Effecto de Factores de Crescentia Super le Crescentia de *Vibrio fetus*

Le effecto de 21 factores de crescentia super le crescentia de *Vibrio fetus* esseva studiate in iste experimento.

Certe concentrationes de cysteina, magnesium, manganese, ferro, lactato, cetoglutarato alpha, glutamato e glutamina, uracil, thymina, acido p-aminobenzoic, e 17(beta)-estradiol promoveva le crescentia de *V. fetus* post lor addition al medio basal.

Le resultatos indica que le lente crescentia de *V. fetus* in medios conventional resulta in parte de certe deficientias in le medios.

Studies on Some of the Variables Associated with the Formation of Plaques by the ECPO-1 Virus

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A PREVIOUS REPORT [10] has indicated that the plaque technique is useful for the study of the porcine enteroviruses. However, it was noticed that certain factors, if not properly controlled, may affect the reproducibility and efficiency of the plaque technique. The aim of this report is to determine the influence of certain variables on the formation of plaques by the ECPO (enteric cytopathogenic porcine orphan)-1 virus on porcine kidney cell monolayers.

Some of the variables affecting the formation or morphologic characteristics of plaques on monolayers of cells have been reported for a number of viruses. Examples are the works of Dulbecco *et al.*[4] and Youngner [12] with polio virus, Bachrach *et al.*[1] with foot-and-mouth disease virus, and Kaplan [7] with herpes simplex virus.

MATERIALS AND METHODS

The methods for preparing mediums, making monolayers of porcine kidney cells, and assaying the virus by the plaque technique were essentially the same as described previously.[5, 10, 11]

The following abbreviations are used: LAHM = lactalbumin hydrolysate medium; BSS = Hanks' balanced salt solution; and PFU = plaque-forming unit.

Monolayer cultures of porcine kidney cells were prepared in 4-oz. prescription bottles.

Plaque Technique.—Monolayer cultures were laid bare by aspiration of growth medium and were infected with 0.2 ml. of virus diluted in BSS to produce about 40 plaques per bottle. The bottles were gently rocked to spread the inoculum evenly over the monolayer culture and were then incubated at 37 C. for 60 minutes, except when otherwise stated, to allow for virus adsorption. Following incubation, the infected monolayers were overlaid with 10 to 12 ml. of agar medium which was prepared as follows: to 100 ml. of BSS was added 0.5 Gm. of lactalbumin hydrolysate and 1.0 Gm. of Noble agar (Difco). This was autoclaved at 7 lb. of pressure for ten minutes and then placed in water bath and cooled to 42 C.; then 3 ml. of bovine serum, 1.5 ml. of a 1:1,000 dilution of neutral red, and 6.0 ml. of 4.4 per cent solution of sodium bicarbonate were added. Antibiotics were also added, giving a final concentration of 100 units of penicillin, 100 μg. of dihydrostreptomycin, and 50 units of nystatin* per milliliter of medium. After gelatination occurred, the culture bottles were inverted and incubated at 37 C.

A porcine enterovirus, designated ECPO-1,[2] was used throughout in this work.

RESULTS AND DISCUSSION

Influence of Time on the Adsorption of Virus.—The following experiments were designed to indicate how rapidly ECPO-1 virus becomes adsorbed to a monolayer of porcine kidney cells.

Each of 20 bottle cultures of porcine kidney cells were washed with BSS at pH 7.4 and inoculated with 0.2 ml. of stock virus diluted in BSS to give approximately 40 plaques per bottle as determined by previous titration of an aliquot of the same pool of virus. Infected cultures were incubated at 37 C. to allow for virus adsorption. At different intervals (table 1), four bottles were removed from the incubator and agar overlay was added immediately to two of them. The remaining two cultures were washed three times, each time using 10 ml. of BSS, and were overlaid with agar. This washing procedure was intended to remove unadsorbed virus.[7] All culture bottles were incubated at 37 C. Plaque counts were made daily from the third to the seventh day of incubation, at which time the experiment was terminated.

The results (table 1) indicate that (1) The rate of virus adsorption is relatively fast. After 15 minutes, approximately 71 per cent of the viable virus particles were adsorbed as compared to adsorption which occurred at 120 minutes. (2) The majority of the unadsorbed virus particles which became entrapped in the agar overlay eventually reached the cellular monolayer and produced plaques. The results of a

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* Mycostatin, produced by E. R. Squibb & Sons, New York, N.Y.

similar experiment (table 2) indicated that, after an adsorption period of 5 minutes at 37 C., approximately 61 per cent of the viable virus particles were adsorbed as compared to adsorption which occurred at 120 minutes.

TABLE 1—Adsorption of ECPO-1 Virus to Monolayer Cultures of Porcine Kidney Cells

Time of adsorption (minutes)	No. of plaques per culture	
	Washed culture*	Unwashed culture
15	20, 21	32, 30
30	20, 23	30, 33
60	26, 28	28, 32
120	27, 29	35, 30
180	28, 27	32, 30

* Washed three times following the virus adsorption period.

The influence of time on the adsorption of ECPO-1 virus to a monolayer culture of porcine kidney cells is similar to the adsorption of poliomyelitis viruses onto monkey kidney cells as reported by Youngner,[12] except that the rate of adsorption of the former virus was more rapid.

Diffusion of Virus Through the Agar Overlay.—As indicated (table 1), the number of plaques occurring in the unwashed cultures was not influenced by the adsorption period. This indicated that the majority of the viable virus particles trapped in the agar overlay eventually reached the monolayer culture and produced plaques.

To further confirm the ability of the ECPO-1 virus to pass through an agar overlay, a more direct experiment was conducted. Five milliliters of the liquid agar medium was added to a culture and allowed to solidify over the cellular monolayer. A stock virus (0.2 ml., diluted to give approximately 50 plaques) was added and evenly distributed on top of the agar. An additional 10 ml. of liquid agar medium was then added on top of the first agar layer. After four days of incubation, the number of plaques which occurred in this bottle was 50 per cent of that which occurred in a control culture, indicating the ability of the virus to pass through the layer of agar medium.

Thus, the ECPO-1 virus resembles poliomyelitis virus[12] in its ability to pass through the agar overlay. In contrast, herpes-B,[12] vaccinia,[12] and foot-and-mouth disease[1] viruses do not pass through the agar overlay. Bachrach *et al.*[1] suggest that the failure to detect diffusion of foot-and-mouth disease virus through agar may be due to its instability at 37 C.

Effect of Temperature on the Adsorption of Virus.—Thirty cultures were allotted to three equal groups. One hour prior to virus inoculation, cultures in each group were incubated at 4 C., 37 C., or 25 C. All

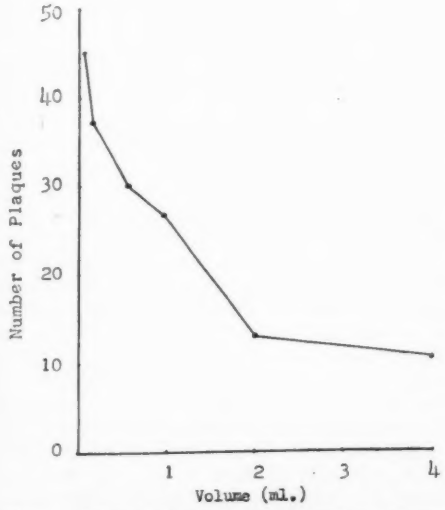

Fig. 1—Effect of volume of diluent on plaque formation by ECPO-1 virus.

TABLE 2—Effects of Time and Temperature on the Adsorption of ECPO-1 Virus

Time of adsorption (minutes)*	No. of plaques per culture		
	37 C.	25 C.	4 C.
5	20, 24	21, 23	16, 17
15	28	24	23, 21
30	31, 31	30, 27	26, 23
60	34, 35	30, 30	32, 30
120	35, 37	32, 34	33, 29

* Cultures were washed after these adsorption periods to remove unadsorbed virus.

TABLE 3—Influence of Normal and Inactivated Bovine Serum in Agar Overlay on Plaque Formation

Bovine serum in agar overlay (%)	No. of plaques per culture	
	Normal serum	Inactivated serum*
0	56, 62	
3	58, 53	60, 65
5	60, 65	55, 61
10	57, 63	61, 65

* Bovine serum was inactivated at 65 C. for 30 minutes.

the cultures were inoculated with a dilution of ECPO-1 virus calculated to produce about 40 plaques per culture, and further incubated at the same temperatures. Following different adsorption periods (table 2) two cultures from each group were washed three times with 10 ml. of BSS (pH 7.4) to remove unadsorbed virus, and then they were overlaid with agar. All cultures were incubated at 37 C.

A slightly greater number of plaques occurred when the temperature at which the virus was allowed to be adsorbed was increased from 4 C. to 37 C., indicating that the rate of virus adsorption is influenced by the temperature (table 2).

These results are similar to those which have been reported for foot-and-mouth disease[1] and poliomyelitis viruses.[12] Bachrach *et al.*[1] state that these results suggest that the primary attachment of virus to a cell layer may be dependent upon chemical or enzymatic reactions with positive temperature coefficients.

TABLE 4—Effect of Various Concentration of Neutral Red in Agar Overlay on Plaque Formation and Size

Neutral red in agar overlay (%)	No. of plaques	Size (ml.)			
		48 hours*	67 hours	96 hours	144 hours
0	14	5	7	12	20
0.001	15	2	4	9	14
0.0015	16	2	4	9	14
0.002	13	2	4	9	12
0.0025	10	2	3	6	8
0.0035	0	0**	0†	0	0

* Hours of incubation after adding the agar.
** Fifty per cent of the cells were dead.
† All cells were dead.

Effect of Volume of Virus Inoculum on Plaque Formation.—Preparations of ECPO-1 virus were made so that the same number of PFU were present in volumes of 0.1, 0.2, 0.6, 1.2, and 4.0 ml. Two cultures, which had been washed once with 10 ml. of BSS in order to remove excess residual nutrient medium, were inoculated with each of these virus preparations. After 30 minutes of incubation at 37 C., as much fluid as possible was aspirated from the bottles and the agar overlay was added.

The plaque count decreased markedly with the increase in volume inoculated (fig. 1); that is, the count dropped from 45 to 11 plaques upon increasing the volume of diluent from 0.1 to 4.0 ml.

Similar results have been reported by Bachrach *et al.*[1] with foot-and-mouth disease virus. Their interpretation, in part, was that the smaller volumes of inoculum provided a more effective collision rate between viruses and cells; this concurs with our explanation.

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Fig. 2—Both bottles were inoculated with the same number of plaque-forming units of ECPO-1 virus. Notice the difference in plaque size, resulting from different concentrations of neutral red in agar overlay.

Effect of Virus Diluent on Virus Adsorption and Plaque Formation.—Two principal virus diluents, BSS and LAHM, were used alone and each with cattle, rabbit, and chicken serum, with serum in amounts of 3 per cent. Thus, a total of eight diluents were tested with each adjusted to pH 7.5. The reference virus was diluted from its original tissue culture state, using these eight diluents. The cultures were washed twice with BSS to remove excess nutrient fluid. Two of these cultures were then used for each diluent and were inoculated with 0.2 ml. of the same dilution of virus. The agar overlay was added after an adsorption period of 30 minutes.

There was no significant difference in the number of plaques arising from the use of these eight virus diluents.

Influence of Concentration of Normal and Inactivated Bovine Serum in Agar Overlay on Plaque Formation.—The monolayer cultures were washed three times with 10 ml. of BSS solution at pH 7.4, in-

fected with a dilution of ECPO-1 virus calculated to give approximately 60 plaques, and allowed to incubate at 37 C. for 60 minutes. At the end of this incubation period, 10 ml. of agar medium, containing varying concentrations of normal or heat-inactivated bovine serum, was added onto each culture.

It was found that increasing the concentration of bovine serum, either normal or inactivated, from 0 to 10 per cent in the agar overlay did not influence the number of plaques obtained, their morphology, or the time of their appearance (table 3). Plaques were well-defined and appeared 48 to 72 hours after virus inoculation. They reached their maximum number and size by the seventh to tenth day. Since increasing concentration of bovine serum from 0 to 10 per cent in agar overlay did not influence the number of plaques or their morphology, the possibility that the serum tested contained ECPO-1 virus inhibitor was excluded.

Henry and Youngner [6] have shown that increasing concentrations of fresh, normal, rabbit serum in the agar overlay medium markedly decreases the number and size of plaques of influenza type A because of a virus-inhibiting substance in fresh rabbit serum. It should be emphasized that only serums which are known to be free of viral inhibitory substances should be used.

Effect of Washing Monolayer Cultures Prior to Virus Inoculation.—Two cultures were washed once with 10 ml. of BSS (pH 7.6). Two similar cultures were left unwashed. Each of the four cultures was then inoculated with the same amount of ECPO-1 virus calculated to yield a countable number of plaques.

A similar number of plaques arose in each culture, indicating that washing the cellular monolayer prior to virus inoculation did not influence the number of plaques that occurred.

Effect of Various Concentrations of Neutral Red in Agar Overlay on Plaque Formation and Size.—Various concentrations of neutral red were incorporated in agar overlay, and their effect on the formation of plaques of ECPO-1 virus was determined. The results are summarized (table 4) and indicate that the percentage of neutral red in the agar overlay may influence the number and size of plaques. At a concentration of 0.0035 per cent, the porcine kidney cells were dead, as determined microscopically and by their inability to take up neutral red stain. There was a difference in plaque morphology when two different concentrations of neutral red were incorporated in agar overlay (fig. 2).

Darnell *et al.*[3] reported that neutral red (1:30,000) in the agar overlay decreased the number, but not the size, of plaques that occurred with type 1 polio virus and Western equine encephalomyelitis virus on HeLa and chicken embryo cell monolayers, respectively. Similarly, McClain and Hackett [8] reported a decrease in the number of plaques (vesicular stomatitis virus) when utilizing neutral red (1:40,000) in the agar overlaying chicken fibroblast monolayers.

Pledger [9] reported that decreasing the neutral red concentration in agar overlay decreased the incubation time necessary for maximal plaque formation by foot-and-mouth disease virus, type A, on primary bovine calf kidney tissue cultures. At a 1:50,000 dilution of neutral red in agar overlay, a quantity sufficient to delineate plaques, no inhibition of plaque formation occurred.

The toxicity of neutral red to the cellular monolayer probably varies, depending on the origin of the cells, and this in turn will greatly determine the concentration of neutral red that can be effectively used in the agar overlay. It seems reasonable to suspect that neutral red influences the size and number of plaques either by increasing the resistance of stained cells to the virus or by acting as a virucidal agent.

SUMMARY AND CONCLUSIONS

Approximately 71 per cent of the viable virus particles of ECPO (enteric cytopathogenic porcine orphan)-1 virus were adsorbed to monolayers of porcine kidney cells in 15 minutes at 37 C., as compared to the number of virus particles adsorbed after an incubation period of 120 minutes.

The majority of unadsorbed virus particles were capable of diffusing through the agar, eventually reaching the cell layers and producing plaques.

There was no significant difference in the rate of virus adsorption to cellular monolayers at 37 C. and 25 C., but there was a decrease in the rate of virus adsorption at 4 C.

Plating efficiency was influenced by the volume of virus preparation placed in contact with the monolayer culture.

Cattle, rabbit, and chicken serums in virus diluents (lactalbumin hydrolysate medium and Hanks' balanced salt solution) did not affect the rate of virus adsorption to the cellular monolayers. Increasing concentrations of normal bovine serum in agar overlay did not affect the number of plaques or their morphology.

Washing cultures before inoculation of virus was not necessary.

The concentration of neutral red in agar overlay was found to be important in determining the size and number of the ECPO-1 virus plaques.

References

[1] Bachrach, H. J., *et al.*: A Plaque Assay for Foot-and-Mouth Disease Virus and Kinetics of Virus Reproduction. Virology, 4, (1957): 224–236.

[2] Bohl, E. H., Singh, K. V., Hancock, B. B., and Kasza, L.: Studies on Five Porcine Enteroviruses. Am. J. Vet. Res., 21, (Jan., 1960): 99–103.

[3] Darnell, J. E., Jr., Lockhart, R. Z., Jr., and Sawyer, T. K.: The Effect of Neutral Red on Plaque Formation in Two Virus Cell Systems. Virology, 6, (1958): 567–568.

[4] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., 99, (1954): 167–182.

[5] Hancock, B. B., Bohl, E. H., and Birkeland, J. M.: Swine Kidney Cell Cultures—Susceptibility to Viruses and Use in Isolation of Enteroviruses of Swine. Am. J. Vet. Res., 20, (Jan., 1959): 127–132.

[6] Henry, C., and Youngner, J. S.: Influence of Normal Animal Sera on Influenza Viruses in Culture of Trypsin-Dispersed Monkey Kidney Cells. J. Immunol., 78, (1957): 273–281.

[7] Kaplan, A. S.: A Study of Herpes Simplex Virus Rabbit Kidney Cell System by Plaque Technique. Virology, 4, (1957): 435–457.

[8] McClain, M. E., and Hackett, A. J.: A Comparative Study of the Growth of Vesicular Stomatitis Virus in Five Tissue Culture Systems. J. Immunol., 80, (1958): 356–361.

[9] Pledger, R. A.: Effect of Neutral Red on Plaque Formation by Foot-and-Mouth Disease Virus. Virology, 10, (1960): 50–56.

[10] Singh, K. V., Bohl, E. H., and Birkeland, J. M.: The Use of Plaque Technique for the Study of Porcine Enteroviruses. Am. J. Vet. Res., 20, (May, 1959): 568–572.

[11] Singh, K. V.: Study of Porcine Enteroviruses by the Plaque Technique. M.S. thesis, The Ohio State University, Columbus, 1958.

[12] Youngner, J. S.: Virus Adsorption and Plaque Formation in Monolayer Cultures of Trypsin-Dispersed Monkey Kidney. J. Immunol., 76, (1956): 288–292.

SUMMARIO IN INTERLINGUA

Studios in Certes del Variabiles Associate con le Formation de Placas per le Virus "ECPO-1"

Approximativemente 71 pro cento del viabile particulas de virus ECPO-1 (E = enteric; C = cytopathogenic; P = porcin; O = orphano) esseva adsorbite a monostratos de cellulas renal de porco in le curso de 15 minutas a 37 C, in comparation con le numero de particulas de virus adsorbite post un periodo de incubation de 120 minutas.

Le majoritate del non-adsorbite particulas de virus esseva capace a diffunder se a transverso le agar e a attinger in le curso del tempore le stratos cellular ubi illos formava placas.

Esseva notate nulle significative differentia in le intensitate del adsorption de virus a monostratos de cellulas inter 37 C e 25 C, sed un reduction de ille intensitate esseva constatate a 4 C.

Le efficacia del formation de placas esseva influentiate per le volumine del preparato de virus que esseva placiate in contacto con le cultura monostratal.

Le addition de sero de bestial, de conilio, e de gallina al diluentes del virus (i.e. un medio de hydrolysato de lactalbumina e un balanciate solution salin de Hanks) non afficeva le adsorption de virus al monostrato de cellulas. Crescente concentrationes de normal sero bovin in le copertura de agar non afficeva le numero o le morphologia del placas formate.

Il non esseva necessari lavar le culturas ante le inoculation de virus.

Esseva constatate que le concentration de rubio neutre in le copertura de agar esseva importante in le determination del dimensiones e del numero del placas de virus.

The Experimental Replacement of Segmental Defects in Bone with a Plaster of Paris-Epoxy Resin Mixture

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THE TREATMENT of diseases and fractures of bone sometimes results in large osseous defects. These defects may not heal (pseudoarthrosis) or, if healing does occur, the animal will have a limb with limited function.

Homogenous or autogenous bone grafts can be used in many instances with a fair degree of success; however, the maintenance of a bone bank or the technology involved in the use of grafts is often beyond the limit of practicality for the general practitioner.

Therefore, it would be desirable if a compatible, inexpensive material were available for use. This material would replace the bone defects, enter into fracture repair (actively or passively), and, in time, be replaced completely by normal bone, thus allowing the return of normal function to the limb.

The use of plaster of paris to fill defects of bone was reported as early as 1928 [4] and 1930.[2]

Peltier *et al.*,[5] studying the use of plaster of paris to fill defects in the bones of dogs, concluded that (1) plaster of paris ($CaSO_4$) was absorbed from the areas of implantation in bone and was well-tolerated by the tissue; (2) in association with the absorption of the plaster, bony regeneration from endosteal and periosteal cells occurred.

Gourley [3] found that a plaster-of-paris column, held in a large femoral segmental bone defect of a dog by a threaded Steinman intramedullary pin, lacked adequate strength to withstand the stresses placed on it. The column broke down within 24 hours after being put in place.

It was thought, therefore, that if the strength of the plaster of paris were increased, it could be used to fill in large osseous defects.

MATERIALS AND METHODS

Commercial grade plaster of paris was mixed with water in the proportions of one-half plaster to one-half water by volume, so that the resulting mixture was quite fluid. To the plaster of paris was added an epoxy resin.* The latter was prepared by mixing by volume equal portions of part A (alloy) to part B (hardner).

Three different mixtures were made: (1) one-fourth part epoxy resin to three-fourths part plaster of paris by volume; (2) one-half part epoxy resin to one-half part plaster of paris by volume; and (3) three-fourths part epoxy resin to one-fourth part plaster of paris by volume.

The mixtures were made by adding the prepared epoxy resin to the prepared plaster of paris; after thorough mixing, they were poured into glass molds and allowed to set for 24 hours. After the mixtures had set, the glass molds were broken away, giving columns 2.5 centimeters in diameter and 10 centimeters long.

Procedure.—Seven mongrel dogs, ages varying from 7 months to 4 years, were used; each weighed approximately 13.6 kg. A radiograph of the right femur was taken prior to surgery to determine the diameter of the intramedullary pin to be used so that a hole of corresponding size could be drilled in the plaster-resin column. The column then was cut to the desired length and, in some instances, the circumference was pared with a knife so that it would more closely fit the actual diameter of the bone. The plaster-resin column was then placed in the instrument tray with the other surgical instruments and autoclaved for at least 45 minutes.

Each animal was anesthetized with pentobarbital sodium given intravenously and the right femoral area was carefully prepared. The incision was made through the skin over the fascia lata at the junction of the biceps femoris caudally and lateral head of the quadriceps femoris cranially. The fascia lata was incised and the two muscles retracted in their respective directions, thus exposing the midshaft portion of the femur. A wire saw was placed around the bone and a complete section of the femur, varying from 3 to 4.5 cm. in length, was removed. The appropriate threaded Steinman intramedullary pin was then forced through the proximal end of the femur

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* Resiweld Plastic Alloy, No. 620, produced by H. B. Fuller Co., St. Paul, Minn.

until the trochar end emerged through the skin over the region dorsal to the coxofemoral joint.

The trochar end of the pin was grasped with the bone chuck and the pin pulled out until the threaded end just protruded from the distal end of the proximal fragment of the femur. The plaster-resin column was then placed over the threaded end of the pin, and the pin was forced through the column until it protruded one-eighth of an inch beyond the other end of the column. The protruding end of the pin was used to catch the medullary cavity of the distal fragment of the femur, then rotated into the distal end of the femur and anchored. The fascia and skin were sutured and radiographs of the leg were made. If the pin were found to be anchored well in the distal end of the femur, it was cut off where it emerged from the coxofemoral area as far down into the trochanteric fossa as possible. No antibioties or supporting splints were used postoperatively.

In that an elevation of the serum calcium would suggest absorption of calcium from the implants, serum calcium levels were followed in these animals. Blood samples for serum calcium determinations were drawn preoperatively on three successive days (to establish normal levels in these animals) and at weekly intervals for the 90-day postoperative period. The serum calcium levels were determined by the Clark-Collip modification of the Tisdall method.[2]

Further postoperative radiographs were taken of the femurs at weekly intervals and at the time of euthanasia, which was three months after the operation. The intramedullary pin in dog 5 was removed 15 days prior to euthanasia to ascertain if sufficient union had occurred to hold up without the intramedullary pin.

After euthanasia, the operative sites were examined grossly and the entire femurs were removed. In dogs 3, 5, and 7, the femurs were placed in 10 per cent formalin; whereas, in dogs 1 and 2, the femurs were sawed in half longitudinally and half was placed in 10 per cent formalin and the other half cleaned and bleached for permanent specimens. The muscle surrounding the operative site in dog 5 also was placed in 10 per cent formalin.

Following fixation of the tissues, bone blocks were removed from the implantation sites and decalcified. Decalcified bone and muscle blocks were sectioned and stained with hematoxylin and eosin and Mallory's triple stain. All stained sections were studied histologically.

RESULTS

The results are summarized (table 1). Only in dog 5 did a draining wound develop, and this was subsequent to the removal of the intramedullary pin and the breaking up of the plaster-resin column.

TABLE 1—Results of Segmental Defects in Bone Replaced with a Plaster of Paris-Epoxy Resin Mixture

Dog No.	Size of defect and replacement column	Composition of column	Length of time column in place	Radiographic changes at 90 days	Gross appearance at 90 days
1	3.0-cm. defect; 3.0 x 2.5 cm. replacement column.	¾ plaster of paris to ¼ epoxy resin	90 days.	No evidence of column absorption. Moderate periosteal reaction tending to surround implant. Ends of femur next to implant broadened.	Partially stable fibrous union.
2	3.0-cm. defect; 3.0 x 2.5 cm. replacement column.	¾ plaster of paris to ¼ epoxy resin.	90 days.	No evidence of column absorption. Extensive periosteal reaction to implant having a rough, cauliflower appearance. Bony reaction extended out, tending to encompass implant.	Unstable fibrous union.
3	4.5-cm. defect; 4.5 x 2.0 cm. replacement column.	½ plaster of paris to ½ epoxy resin.	90 days.	No evidence of column absorption. Extensive periosteal reaction up and down entire femur, smooth in appearance. Diameter of femur doubled in size. Bone tended to encompass implant.	Unstable fibrous union.
4	4.5-cm. defect; 4.5 x 2.0 cm. replacement column.	½ plaster of paris to ½ epoxy resin.	Less than 24 hours.	Column broke down within 24 hours and dog was destroyed.	No evaluation.
5	4.0-cm. defect; 4.0 x 2.0 cm. replacement column.	¼ plaster of paris to ¾ epoxy resin.	90 days; intramedullary pin removed at 75 days.	No evidence of column absorption. The cut ends of the femur impacted into implant at 75 days, with bone growth extending partially around the column. After removal of the pin, column fragmented.	Unstable fibrous union.
6	3.0-cm. defect; 3.0 x 3.0 cm. replacement column.	Epoxy resin only.	15 days.	Dog died; distemper convulsions.	No evaluation.
7	3.0-cm. defect; 3.0 x 3.0 cm. replacement column.	Epoxy resin only.	90 days.	No evidence of column absorption. Minimal periosteal reaction. Some bony growth tending to encompass implant.	Unstable fibrous union.

Fig. 1—Lateral view of sagitally cut femurs of dog 1 (left) and dog 2 (right). There is a thin, tight, fibrous capsule around the implant in dog 1. In dog 2, notice the extensive cauliflower-like periosteal reaction and the bone tissue starting to encompass the implant. The intervening space between the bony outgrowths was filled with a thick, loose, connective-tissue capsule.

The suppurative tract led directly to the implantation site.

With the pin and column in place, the animals used the treated legs to some degree. In fact, in dogs 1, 2, and 3 there was little evidence of lameness after they had been allowed to exercise for an extended period of time.

There was no complete bony union in any of the animals. Dog 1 had a firm, fibrous union (with some bone contributing) around the column, but it is questionable how it would have stood up had the intramedullary pin been removed. (fig. 1, 2).

There was no indication of absorption of the columns, either radiologically, grossly, or histologically. The columns acted as foreign bodies; the new bone formation tended to surround the plaster-resin and the ends of the bone next to the implant broadened. The degree of bone response to the implant seemed to be proportional to the osteogenic capabilities of the animal involved. Dog 3, the youngest (7 months of age), had the greatest degree of osteogenic reaction.

Histologically, in all cases there was osseous trabecular proliferation at the ends of the bone adjacent to the implant. There were no bony infiltrations into the plaster-resin columns. Macrophages with ingested plaster-resinous material were present in all sections, as were varying numbers of plasma and other mononuclear-type cells. In dog 5, there was a heavy infiltration of polymorphonuclear cells in the muscle around the implantation site, with foreign-body giant cells observed in addition to

Fig. 2—Medial view of the specimens shown in figure 1. Notice again the thin, tight, fibrous capsule in dog 1 (left) and the bony proliferation in dog 2 (right). Also notice the broadening of the cut ends of the femurs next to the implants. In dog 2, it appears that there was anterior-posterior movement of the femur on the pin, since the medullary cavity is broadened in that direction. Special attention should be paid to the neck and head of the femurs. As the pins were inserted through the upper segments of the femurs, the heads and necks were damaged. This should be remembered when employing this method of pinning.

the plasma cells, mononuclear cells, and macrophages. There was extensive muscle necrosis, and, in some areas, no viable striated muscle fibers were visible, having been replaced by the cellular infiltration.

Serum calcium determinations did not vary significantly from the considered normal value of 8 to 10 mg./100 cc. of serum.

SUMMARY

When a plastic alloy was added to plaster of paris, it substantially increased the strength of the latter, enabling it to withstand the stresses encountered when used as a replacement column for segmental defects in the femurs of dogs. The epoxy resin, however, made the plaster of paris incompatible with the tissues in the 5 dogs completely studied. The incompatibility was evidenced grossly and radiographically by nonunion of the fractures, and microscopically by the chronic inflammatory response.

References

[1] Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium with a Suggested Modification. J. Biol. Chem., 63, (1925): 461.

[2] Edberg, E.: Some Experiences of Filling Osseous Cavities with Plaster. Acta chir. scandinav., 67, (1930): 313.

[3] Gourley, I. M. G.: Unpublished data, University of Minnesota, St. Paul, 1958.

[4] Nystrom, G.: Plugging of Bone Cavities with Rivanol-Plaster-Porridge. Acta chir. scandinav., 63, (1928): 296.

[5] Peltier, L., Bickel, E., Lillio, R., and Thein, M.: The Use of Plaster of Paris to Fill Defects in Bone. Ann. Surg., 146, (1957): 61.

SUMMARIO IN INTERLINGUA

Le Reimplaciamento Experimental de Defectos Segmental de Osso per un Mixtura de Gypso e Epoxy-Resina

Quando un alligato de plastico esseva addite a gypso, illo augmentava considerabilemente le fortia del gypso, rendente lo capace a resister al stresses incontrate quando le mixtura esseva usate como columna de reimplaciamento pro defectos segmental in le femore del can. Tamen, le epoxy-resina rendeva le gypso incompatibile con le tissus de 5 canes le quales esseva studiate completemente. Le incompatibilitate esseva evidentiate grossiermente e radiographicamente per non-union del fracturas e microscopicamente per un chronic responsa inflammatori.

Scientific Notes

Erythrocyte Survival Studies in the Horse, Using Glycine-2-C^{14}

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GLYCINE-2-C^{14} has been previously used in studies concerning the life span of red blood cells in man,[7] rats,[1] swine,[8] and various zoo species.[4] In the present study, equine erythrocytes were labeled in vivo by intravenous injection of glycine-2-C^{14}, from which the α-methylene carbon atom is incorporated into eight of the 34 carbon atoms of the porphyrin moiety of hemoglobin. Survival times of C^{14}-labeled erythrocyte populations were previously described[4] by giving corresponding survival probability functions, p(t), where p(t) is the probability that an erythrocyte will have a survival time greater than t. This information was displayed graphically and summarized by giving, for each erythrocyte population, the median survival time, $t_{1/2}$, a quantity which is defined by the equation $p(t_{1/2}) = \frac{1}{2}$. The term "median" has a standard statistical meaning and appears to be a better term than "half-survival time"[4] or "half-time," since the latter are used solely for the median of an exponential distribution. In the present investigation, the median survival times of erythrocyte populations in two Thoroughbred stallions were measured for the first time using carbon-14.

MATERIALS and METHODS

Two Thoroughbred stallions (59-1 and 59-2) were inoculated intravenously with 2.0 and 5.0 μc./kg., respectively. Stallions 59-1 and 59-2 (7 and 2 years of age) weighed 486 and 503 kg., respectively. Erythrocyte counts were performed weekly. Hemin was isolated from 20 ml. of venous blood,[5] then recrystallized,[7] combusted to CO_2, and prepared for radioactive assay.[4] No correction was made for loss of radioactivity removed by sampling, since it contributed to error less than that encountered in counting samples for radioactivity. Probabilities of erythrocyte survival and half, or median, survival times in the 2 stallions were calculated by methods previously reported.[4]

RESULTS

Graphs of the survival probability functions, p(t), for labeled erythrocyte populations are shown as dotted lines (fig. 1). Median survival times

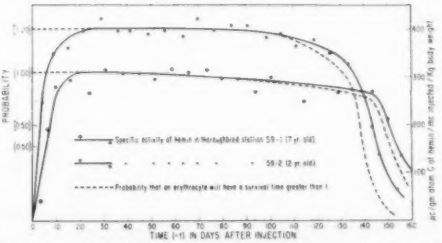

Fig. 1—Probability of erythrocyte survival and specific activity of hemin in 2 Thoroughbred stallions following intravenous injection of glycine-2-C^{14}

of the C^{14}-labeled erythrocyte populations in stallions 59-1 and 59-2 were approximately 150 and 140 days, respectively. It would appear from this study that mammalian erythrocytes of the family Equidae may have survival times similar to, or possibly slightly longer, than that observed in man (110 to 130 days).[2] This is in contrast to the findings of Hansard and Kincaid,[6] in which the life span of erythrocytes in burros was estimated to be less than 60 days.

SUMMARY

Using glycine-2-C^{14}, erythrocyte survival was studied in 2 horses. Median survival times for C^{14}-labeled erythrocyte populations were approximately 140 and 150 days.

References

[1] Berlin, N. I., Meyer, L. M., and Lazarus, M.: Life Span of the Rat Red Blood Cell as Determined by Glycine-2-C^{14}. Am. J. Physiol., 165, (1951): 565.

[2] Berlin, N. I., Woldmann, T. A., and Weissman, S. M.: Life Span of the Red Blood Cell. Physiol. Rev., 39, (1959): 577.

[3] Bush, J. A., Berlin, N. I., Jensen, W. V., Brill, A. B., Cartwright, C. E., and Wintrobe, N. M.: Erythrocyte Life

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Span in Growing Swine as Determined by Glycine-2-C^{14}. J. Exptl. Med., 101, (1955): 451.

[4] Cornelius, Charles E., Kaneko, Jiro J., and Benson, D. C.: Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok Antelope, Using Glycine-2-C^{14}. Am. J. Vet. Res., 20, (Sept., 1959): 917.

[5] Fisher, H.: Hemin. Org. Synthesis, 21, (1941): 53.

[6] Hansard, S. L., and Kincaid, E.: Red Cell Life Span of Farm Animals. J. Anim. Sci., 15, (1956): 1300.

[7] Shemin, D., and Rittenberg, D.: The Life Span of the Human Red Blood Cell. J. Biol. Chem., 166 (1946): 627.

SUMMARIO IN INTERLINGUA

Studios del Superviventia de Erythrocytos Equin, con le Uso de Glycina-2-C^{14}

Con le uso de glycina-2-C^{14}, le superviventia del erythrocytos esseva studiate in vivo in 2 cavallos. Le tempores median del superviventia de populationes de erythrocytos marcate per glycina-2-C^{14} esseva approximativemente 140 e 150 dies, respectivemente.

Electrophoretic Study of Time-Temperature Effect on Swine Serum

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THE albumin-gamma globulin complex (AGGC) formed in swine serum by the action of heat has been shown previously to be toxic [1] and separable, by isoelectric precipitation at pH 5.5, from low ionic strength solution.[2] Certain similarities can be shown between the AGGC of swine serum and the "C" substance from heated horse serum.[3] Both are colloidal in nature, can be removed from the remaining serum proteins by high speed centrifugation, can be removed from suspension by dialysis against distilled water followed by adjustment to pH 6 for hose serum and pH 5.5 for swine serum, and have electrophoretic heterogeneity in that the separated materials, when resuspended in saline solution, have broad, irregular, electrophoretic patterns. The purpose of this report is to show the time-temperature relationship for the formation of AGGC in swine serum.

Swine serum 638 consisted of pooled samples from 40 nonvaccinated, 50-lb. swine. Ten-milliliter samples of this serum were placed in 26 by 110 mm. screw-capped vials. Approximately two-thirds of the vial was immersed in a water bath at the specified temperature. Stirring was accomplished by slowly rotating the vial about its longitudinal axis. Five minutes was required to bring each sample to the specified temperature. Samples were heated to 60 C. for 30 minutes and one, two, four, eight, and 16 hours; to 62 C. for one, two, four, and eight hours; to 64 C. for one, two, four, and eight hours; and to 66 C. for one, two, and four hours. At the conclusion of the exposure to heat, samples were chilled in running tap water. One sample was exposed at each time-temperature.

After heat treatment, samples were diluted with four volumes of phosphate buffer, pH 7.6, and dialyzed against the same buffer for 16 hours. Analyses were made in an Aminco portable electrophoresis apparatus, using a current of 26 ma. for two hours. Final patterns were recorded photographically, enlarged, and the areas measured planimetrically. Percentages of serum components were calculated. The amount of AGGC was obtained by subtracting the areas of the α and β globulins in the unheated serum from the broad, unresolved, corresponding area in the heated serums. This does not take into account any

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changes occurring to the α and β globulins during heating, but does give a reasonable approximation of the amount of AGGC formed.

The electrophoretic analyses of these samples indicated a progressive increase of AGGC with increase of temperature for any given time interval. There was a similar increase of AGGC with increased time of heating at a given temperature. Upon examination of the electrophorographs, no difference was seen in the nature of AGGC formed under the influence of extended time or higher temperature. The only significant change was in the amount of AGGC (table 1). Each value in table 1 is the percentage of AGGC formed in one sample of serum during exposure to the combination of time and temperature. The increase in amount of AGGC due to an increase of 2 C. was approximately the same as the increase in AGGC due to doubling the time of heating. For example, 62 C. for one hour produced 20.5 per cent AGGC, 64 C. for one hour produced 28.2 per cent AGGC, and 62 C. for two hours produced 30.1 per cent AGGC. This relationship held throughout the range of time and temperature studied.

TABLE 1—Per Cent of Albumin-Gamma Globulin Complex Formed in Swine Serum Due to Heating

Time (hours	Temperature (C.)			
	60	62	64	66
0.5	8.5	.....	.....	.....
1.0	12.0	20.5	28.2	36.2
2.0	21.6	30.1	34.4	C
4.0	24.0	34.6	C	C
8.0	34.9	C	C	.....
16.0	C	.....	.....	.....

C = Congealed.

The previous demonstration[1] of greater toxicity of serums heated to higher temperatures led to speculation concerning the reason for this increased toxicity. Two alternatives seemed possible. Either larger amounts of AGGC were present in serums heated to higher temperatures or a different complex was formed at higher temperatures. In the present work, it has been shown that larger amounts of AGGC are formed at higher temperatures or extended times of heating. There may be differences between the complexes formed; immunologic analysis of these complexes might clarify this area of doubt.

References

[1] Mathews, J., and Buthala, D. A.: Toxicity of Heated Swine Serum. Am. J. Vet. Res., 19, (Jan., 1958): 32–36.

[2] Mathews, J., and Buthala, D. A.: Hog Cholera Protection Tests with Swine Serum Fractions. 1. Hyperimmune Hog Cholera Antiserum. Cornell Vet., 47, (1957): 179–187.

[3] Van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. L.: An Electrophoretic Study of Heated Horse Serum. J. Immunol., 40, (1941): 39–45.

SUMMARIO IN INTERLINGUA

Studio Electrophoretic del Effecto de Temperatura e Tempore Super le Sero del Porco

Le complexo de albumina e globulina gamma que es formate in sero porcin sub le influentia de alte temperaturas e que—secundo un previe studio—possede proprietates de toxicitate esseva studiate electrophoreticamente post varie periodos de incubation (ab 30 minutas ad 16 horas) a varie temperaturas de incubation (ab 60 C ad 66 C).

Le technica usate es describite.

Esseva constatate que le quantitate del complexo cresce si ben con le tempore como etiam con le temperatura. Le effecto de un augmento del temperatura per 2 C esseva approximativemente identic con le effecto de un duplation del periodo de incubation.

Si o non le complexo es semper le mesme, sin reguardo a qual combination del duo factores de temperatura e tempore es responsabile pro le generation de illo, es forsan clarificabile per studios immunologic.

Thymectomy of the Calf

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THYMECTOMIES in man have been described by Blalock et al.[1] and Orr [2] for removal of tumors and treatment of myesthenia gravis. Thymectomies have been performed on guinea pigs, mice, rabbits, and rats, but have not been reported for domestic animals. The purpose of this report is to describe a method of thymectomy of the calf (Bos taurus).

Anatomy.—The calf has both cervical and thoracic thymic tissue. Sisson and Grossman [4] give a detailed description of the anatomy. At the period of its greatest development, the thymus in the calf occupies the greater part of the anterior mediastinal space, extending caudad to the pericardium, pulmonary artery, and aortic arch. The right and left cervical lobes form the bulk of the organ, extending along the trachea and the esophagus from the thoracic inlet to the thyroid gland.

MATERIALS and METHODS

General.—Ten calves were subjected to thymectomy. Leukocyte counts, differential leukocyte counts, and packed cell volumes were determined on the surgical subjects and controls at weekly intervals starting two weeks prior to surgery. Postoperative observations and body temperatures were recorded daily. In the early cases, penicillin and streptomycin were administered postoperatively. At necropsy, any tissue which appeared similar to thymic tissue grossly was examined histologically.

Early Procedure.—Atropine sulfate (0.02 mg/lb) was injected subcutaneously half an hour before anesthesia. Pentobarbital sodium was administered intravenously (to effect) to produce anesthesia. An average of 500 mg. was required.

The calf was placed on the table in dorsal recumbency as soon as it had been anesthetized. All four legs were stretched caudad and tied to the table. The head was extended forward and secured to the table with a gauze bandage fastened around the mandible.

An incision was made on the midline from the thyroid gland to the manubrium sterni. The sternothyro-hyoid, sternomastoid, and sternocephalic muscles were separated on the midline and retracted to expose the cervical part of the thymus and the trachea, esophagus, carotid arteries, external jugular veins, and vagosympathetic trunks. By blunt and sharp dissection, the anterior extent of each lobe of the thymus was carefully freed, then the dissection was continued back to the bifurcation of the gland and on to the thoracic inlet. An average of 15 vessels required ligation during the dissection of the gland from surrounding structures. Two fingers were forced through the thoracic inlet and, by careful manipulation, as much as possible of the thoracic thymus was "teased" through the inlet.

The cervical muscles were brought into apposition with continuous No. 1 chromic gut sutures and the skin was closed with silk sutures.

Improved Procedure.—It was decided that a more satisfactory anesthetic method was needed, and that the thorax would have to be opened if all of the thymus were to be removed (Table 1).

Preanesthetic atropine was given. A length of polyethylene tubing (PE 190 *) was inserted through a 14-gauge, thin-walled needle ** into the jugular vein so injections could be given easily. An average of 2 ml. of 7 per cent chloral hydrate per pound of body weight was injected to produce light surgical anesthesia. Thiopental sodium was injected as needed to maintain anesthesia. After the animals were tied on the table, they were intubated and the cuff on the endotracheal tube was inflated. Immediately prior to opening the thorax, a Seeler Respirator was attached to the endotracheal tube and artificial respiration was started, using 100 per cent oxygen.

The operation proceeded as described previously, except that the skin incision was continued caudad over the sternum. After the cervical lobes of the thymus had been dissected free, the sternum was divided on the midline with a Satterlee saw. Two fingers were put into the thoracic inlet to protect the great vessels from the tip of the saw which was directed cranially and dorsally. The divided sternum was then forced apart and the ventral pleura was cut if it had not been severed by the

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The technique was developed by the authors for studies reported in part, in a thesis submitted by Dr. Papp in partial fulfillment of the requirements for the Ph.D. degree.

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* Clay Adams, Inc., New York, N. Y.

** Randal Faichness Corp., Boston, Mass.

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TABLE 1—Results of Bovine Thymectomy (Early Procedure)

Animal No.	Breed	Age (days)	Weight (lb.)	Gm. of thymic tissue removed	Gm. of thymic tissue found at necropsy (2 mo. after surgery)	Remarks
1	Holstein-Friesian	10	50	60	28	Remained in deep anesthesia for 24 hours.
6	Guernsey	14	65	65	0	Although anesthesia was light during surgery, the calf remained comatose for 24 hours.
3	Holstein-Friesian	14	67	11	75	The calf remained comatose for 24 hours after surgery.

saw. The thoracic part of the thymus was then dissected from surrounding structures, using an Allis forceps and curved Mayo scissors. Care was taken to not damage the left vagus nerve which was usually surrounded by thymus. In several cases, the gland had to be removed in small fragments since it was difficult to dissect free from the vagus and anterior dorsal thorax. Little hemorrhage occurred in the thorax.

Large, 3/8-circle, cutting needles were used to place 18-gauge stainless steel wire sutures through or around the divided sternebrae to bring them into apposition. The remainder of the closure was similar to that described for the earlier procedure, except that the air in the thorax was evacuated by inflating the lungs prior to finishing closure of the muscle layer. The minimum time in which the actual surgery was accomplished by a surgeon, one assistant, and an anesthetist was 86 minutes.

RESULTS and DISCUSSION

None of the calves surviving the surgery developed a temperature rise or any sign of infection at the surgical site. The white blood counts, differential counts, and packed cell volumes gave no indication of infection. There was no evidence of infection at the necropsies.

Detailed results of the effects of thymectomy on blood count, growth rate, serum electrolytes, and organ weights will be presented elsewhere.[8]

In order to insure removal of all of the thymus tissue in the calf, the sternum should be split and dissection should be meticulous. The place at which thymic tissue is most likely to be left is the thorax, especially the anterior dorsal part.

TABLE 2—Results of Bovine Thymectomy (Improved Procedure)

Animal No.	Breed	Age (days)	Weight (lb.)	Gm. of thymic tissue removed	Gm. of thymic tissue found at necropsy	Remarks
2 *	Angus	14	60	58	0	Died ten hours after surgery due to surgical shock and prolonged anesthesia.
K 1	Angus	17	73	....	....	Endotracheal tube cuff failed and and the calf died before correction could be made.
K 2	Hereford	24	72	....	....	The anterior vena cava was cut during the intrathoracic dissection. Death was due to hemorrhage.
S 1	Shorthorn	53	101	187	0	Necropsy six months after surgery.
H 1	Hereford	129	251	185	7	Necropsy six months after surgery; thymic tissue was found at the apex of the right cervical lobe.
P 1	Holstein-Friesian	12	85	245	0 **	Animal being used for further study.
115	Holstein-Friesian	80	202	179	0 **	Animal being used for further study.

The first 3 calves listed (2, K 1, and K 2) were the first to be subjected to the improved procedure. The calves which survived were able to stand three to four hours after surgery.
* Pentobarbital sodium used. All other calves were given chloral hydrate followed by thiopental sodium.
** Blood counts indicate that probably no thymic tissue was retained.

SUMMARY

Thymectomy was performed on 10 calves. No infection resulted from the surgery. Three of the

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operations were done without opening the thorax (splitting the sternum). At necropsy two months after surgery, thymic tissue was found in the thoraces of 2 of the calves but none was found in the remaining calf.

Of the 7 calves in which the sternum was split, 1 died due to surgical shock and prolonged anesthesia. One died due to failure of the endotracheal tube cuff, and 1 died from hemorrhage due to the accidental cutting of a thoracic vessel. In 2 of the calves necropsied six months after surgery, no thoracic thymus could be found, but a small mass of thymic tissue was found in the cervix of 1 of them. Further studies are being done on the remaining 2 calves; blood counts indicate that they probably have no retained thymic tissue.

References

[1] Blalock, A., Mason, M. F., Anorgan, H. J., and Riven, S. S.: Myasthenia Gravis and Tumors of the Thymic Region, Ann. Surg., 110, (1939): 544–545.

[2] Orr, T. G.: Operations of General Surgery. 2nd ed., W. B. Saunders Co., Philadelphia, (1949): 713.

[3] Papp, E., and Venzke, W. G.: Effect of Thymectomy in Rats and Calves. In press.

[4] Sisson, S., and Grossman, J. D.; The Anatomy of Domestic Animals. 4th ed., W. B. Saunders Co., Philadelphia, (1953): 554–555.

SUMMARIO IN INTERLINGUA

Thymectomia del Vitello

Thymectomia esseva effectuate in 10 vitellos. Nulle infection resultava ab le operationes. In 3 del casos, le thymectomia esseva executate sin aperir le thorace (i.e. sin finder le sterno). In 2 del 3, sed non in le tertie, le necropsia duo menses post le operation revelava le presentia de tissu thymic in le thorace.

Quanto al 7 vitellos in que le sterno esseva findite, 1 moriva in consequentia de choc chirurgic e del prolongate anesthesia, 1 moriva a causa de malfunction del manchetta del tubo endotracheal, e 1 moriva per le hemorrhagia initiate per le dissection accidental de un vaso thoracic. In 2 del remanente 4, necropsia sex menses post le chirurgia revelava nulle thymo in le thorace, ben que in le 1, un micre massa de tissu thymic esseva trovate in le cervice.

Resta 2 vitellos. Illos es usate in studios additional nunc in progresso. Numerationes de cellulas sanguinee indica que illos probabilemente ha retenite nulle tissu thymic.

Response of Hemoglobin and Cellular Elements of Blood After Hypervitaminosis D in Monkeys

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THE monkey colony maintained by the Radiobiological Laboratory was accidentally exposed to acute hypervitaminosis D. Excessive amounts of vitamin D, accompanied by elevated calcium and phosphorus content in the ration, were ingested for nearly three months. This episode and its clinical and pathologic effects have been described.[2] It was of practical importance to determine whether survivors were hematologically normal. Two criteria were used: (1) comparison of hematologic values of monkeys before the episode and 12 to 16 months afterwards, and the relationship of these values to reported normal ranges; and (2) comparison of the results of experimental hemorrhage before and after the intoxication. Statistical analysis has been extensively used to test the data. The examination of the Macaca mulatta by Krise and Wald [5,6] in this laboratory afforded information on the normal hematologic values and the response to phlebotomy prior to the vitamin D exposure.

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The authors thank Robert Riess, Lillian Jordan, Barbara Olberding, and Shirley Behrman for technical assistance.

TABLE 1—Comparative Peripheral Hematology in Groups of Maca Mulattas

Blood values	Unit	Mean data for groups Ia, Ib, and II: No. of observations	Mean	S.D.	Extremes	Krise and Wald[2]: No. of observations	Mean	S.D.	Extremes	* Gardner[5]: Mean	Extremes
Erythrocytes	cells x 10^6	218	5.58	0.37	4.17– 7.00	876	5.57	0.73	3.11– 8.57	5.28	3.55– 7.66
Hemoglobin	Gm./100 cc.	208	11.3	0.5	9.0 –12.9	655	11.7	3.0	7.0 –16.5	12.6	9.2 –17.0
Packed Cell Volumes	per cent	228	40.0	1.8	32.5 –47.5	392	37.0	6.8	12.0 –51.0	42.0	31.8 –55.8
Leukocytes	cells x 10^3	248	9.10	1.9	4.35–19.45	891	15.10	5.98	1.20–43.10	15.55	5.00–20.50
Polymorphonuclear neutrophils	per cent	248	31.0	14.7	4.0 –82.0	738	35.8	16.7	4.0 –91.0	43.5	35.8 –51.4
Lymphocytes	per cent	248	63.7	15.8	15.0 –96.0	738	60.5	17.3	7.0 –95.0	52.1	42.2 –60.1
Monocytes	per cent	248	2.3	1.9	0.0 – 5.0	738	0.72	0.38	0.0 – 8.0	1.77	0.8 – 3.0
Eosinophils	per cent	248	2.2	1.9	0.0 –15.0	738	2.63	2.37	0.0 –16.0	2.26	0.5 – 4.9
Reticulocytes	per cent	248	0.43	0.32	0.0–216.0	76	0.35	0.19	0.1 – 3.4	1.04	0.0 – 4.0

* Number of observations and standard deviation were not given.

METHODS

Thirty male monkeys (Macaca mulatta), 4 to 12 years old, were tested in five groups, Ia, Ib, II, A, and B. There were 5 monkeys in each group except II, which consisted of 10 monkeys. Those in groups A and B were subjected to two series of blood withdrawals; 4 ml. of blood was obtained weekly for six weeks; then 4 ml. was obtained daily for two five-day periods with two days intervening. Standard procedures[2] were used for preparing and counting blood samples. The monkey colony is maintained in accordance with procedures outlined by Young.[9]

The treatment by subject design for variance analysis by Lindquist[5] was used to asses the effects of the blood aspiration. Significant time differences were further tested for trends by means of linear regression-fitting techniques. The "0.01" level was designated as the requirement for statistical significance; a significant time trend was the ultimate criterion for a biological effect.

RESULTS

Table 1 shows the comparison of hematologic values for groups Ia, Ib, and II (combined since group means were not significantly different) with the results obtained by Krise and Wald[4] on 538 monkeys tested prior to the hypervitaminosis D episode and the values given by Gardner[3] in a review of the literature on the hematology of Macaca mulatta.

Table 2 summarizes the results of the statistical analyses. These are the probabilities that the sample-time means could differ as they do by chance alone under the hypothesis of no time difference. If there were no changes in time except for the effect of hemorrhage, then there probably was an effect due to loss of blood. Monkeys in groups Ia, Ib, and II were not affected within 30 days by 12 hemorrhages.

The response of group A monkeys (fig. 1) to weekly sampling was greater than that of group B monkeys (fig. 2). The hemoglobin values reflected a significant time difference under these conditions; the time trends were not significant.

The hemorrhage schedule of ten samples in 12 days has been referred to as "daily." The results of the daily hemorrhage schedule for group A and B monkeys is summarized (fig. 3). In group A, there was a highly significant trend toward lower levels in all parameters. In group B, similar trends were present but were less pronounced, being significant only in the case of the hematocrit values.

DISCUSSION

Anemia, as indicated by significantly depressed erythrocyte counts and lowered hemoglobin values, is the hematologic finding reported in monkeys[6,7] and in human beings that have experienced acute vitamin D intoxication. Results of this study indicate that 15 months following the hypervitaminosis D incident, animals in the five groups tested were not anemic and their hemoglobin values were within the normal range.

TABLE 2—P Values for Analysis of Variance on Blood Samples Taken Weekly and Daily from Monkeys

Values	Weekly Samples: Group A	Weekly Samples: Group B	Daily Samples: Group A	Daily Samples: Group B
Erythrocytes	$0.05 > P > 0.025$	$0.025 > P > 0.01$	$P < 0.01$	$0.025 > P > 0.01$
Leukocytes	$0.025 > P > 0.01$	Not significant	$P < 0.01$	$0.10 > P > 0.05$
Hemoglobin	$P < 0.01$	$P < 0.01$	$P < 0.01$	$0.05 > P > 0.01$
Packed Cell Volume	$0.05 > P > 0.01$	$0.05 > P > 0.01$	$P < 0.01$	$P < 0.1$

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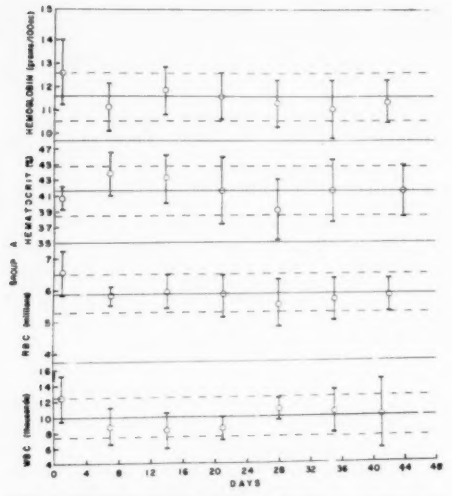

Fig. 1—Effect of weekly hemorrhage on monkeys in group A.

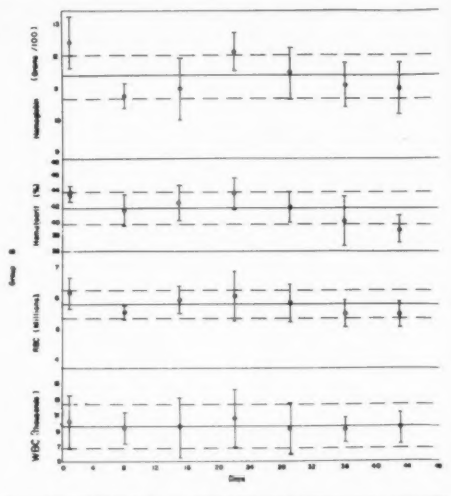

Fig. 2—Effect of weekly hemorrhage on monkeys in group B.

The response of our animals to experimental hemorrhage further supports the belief that they had recovered. Krise and Wald[3] reported that 5, 10, or even 20 ml. of blood obtained weekly from monkeys over a period of eight weeks has little effect on the peripheral blood count. In groups A and B, five consecutive hemorrhages constituted an actual weekly blood loss of 20 ml. The depleting effect of daily hemorrhages was apparent to varying degrees in all observed parameters. The failure of group B monkeys to respond as much as those in group A was probably due to the fact that the former weighed more (mean difference, 1.5 lb.) and thus had greater blood volumes. In light of the findings of Krise and Wald,[3] the possibility cannot be ruled out that these animals, although not anemic, may be more susceptible to phlebotomy. However, successive hemorrhages of 4 ml. would probably cause less stimulus to erythropoietic centers than would one 20-ml. hemorrhage with a six-day "response" time. Since no hemoglobin values were displaced from the normal range, the highly significant time difference was, we feel, due to the "cyclic," or sine wave periodicity, present in the data. Other hemoglobin values in monkeys have indicted similar variation[6,7] which appears to occur within the 95 per cent confidence limits of the mean.

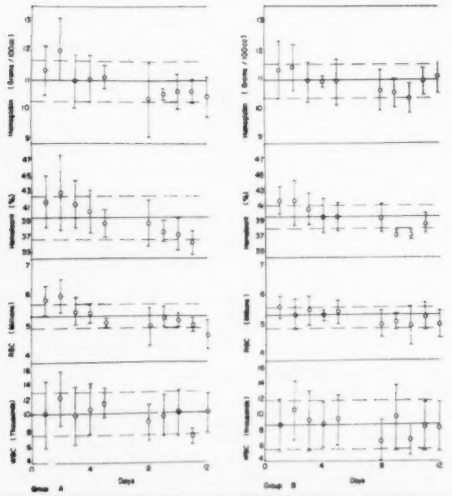

Fig. 3—Effect of daily hemorrhage on monkeys in groups A and B.

The lower white blood cell count in animals which experienced hypervitaminosis D does not appear to have caused increased susceptibility to infection. Values are within the normal range given in the literature for Macaca mulatta.[4] Indeed, in other control groups examined prior to the hypervitaminosis D episode, leukocyte values were in the range of 11,500 cells/cmm.[6]

References

[1] Gardner, M. V.: The Blood Picture of Normal Laboratory Animals. A Review of the Literature, 1936-1946. J. Franklin Inst., 244, (1947): 151–161.

[2] Kent, S. P., Vawter, G. F., Dowben, R. M., and Benson, R.E.: Hypervitaminosis D in Macaca mulatta Monkeys: A Clinical and Pathologic Study. School of Aviation Medicine, USAF, Report No. 57–145, Sept., 1957.

[3] Krise, G. M., Jr., and Wald, Niel: Hematological

Effects of Acute and Chronic Experimental Blood Loss in the Macaca mulatta monkey. Am. J. Vet. Res., (Nov., 1959): 1081–1085.

[6] Krise, G. M., Jr., and Wald, N.: Normal Blood Picture of the Macaca mulatta monkey. J. Appl. Physiol., 12, (1958): 482–484.

[5] Lindquist, E. F.: Design and Analysis of Experiments in Psychology and Education. Houghton Mifflin Co., Boston, 1953.

[6] Melville, G. S., Jr.: Unpublished data, Austin, Texas.

[7] Pickering, J. E., Krise, G. M., Jr., Overall, J. E., Borella, H., Young, R. J., and Williams, C. M.; Effects of Chronic Radiation on the Peripheral Blood Elements of The Monkey (Macaca mulatta): Neutron radiation (first 12 months). In preparation.

[8] Scharfman, W. B., and Propp, S.: Anemia Associated With Vitamin D Intoxication. New England J. M., 255, (1956): 1207–1212.

[9] Young, R. J., Fremming, B. D., Benson, R. E., and Harris, M. D.: Care and Management of a Macaca mulatta Monkey Colony. Proc. Animal Care Panel, 7, 1957.

SUMMARIO IN INTERLINGUA

Responsa de Hemoglobina e de Elementos Cellular del Sanguine post Hypervitaminose D in Simias

Le colonia de macacas mulatte al Laboratorio Radiobiologic de Austin, Texas, esseva accidentalmente exponite a acute hypervitaminose D. Iste episodio e su effectos clinic e pathologic es describite. Duo criterios esseva usate pro determinar si le superviventes esseva hematologicamente normal: (1) Le comparation del valores hematologic registrate pro le macacas ante le episodio con le correspondente valores inter 12 e 16 menses post illo e (2) le comparation del resultatos de hemorrhagias experimental ante le episodio de intoxication con tal resultatos post ille episodio.

Tanto pro simias como etiam pro humanos reportos in le litteratura indica que le experientia de un acute intoxication per vitamina D resulta in anemia, manifeste in significativemente reducite numerationes erythrocytic e nivellos de hemoglobina. In le hic reportate caso, le studio del macacas 15- menses post le incidente de hypervitaminose D revelava que le animales non esseva anemic in ulle del cinque gruppos testate e que lor valores pro hemoglobina esseva intra le limites del norma. Etiam le responsa del animales a hemorrhagia experimental indica que illos se habeva restablite.

Il pare que le reducite numeration leucocytic in le animales exponite a hypervitaminose D non augmentava lor susceptibilitate pro infectiones.

Serologic Relationship Between Bovine Enteroviruses and Infectious Bovine Rhinotracheitis Virus

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THE ISOLATION and preliminary characterization of several bovine enterovirus (BEV) isolates has been reported previously.[1,2] In the majority of the cattle herds in which BEV cultures were isolated, there were histories of acute but mild respiratory disease among calves, particularly during the weaning period. The clinical manifestation consisted of fever (103–106 F.), slight nasal exudate, and, in some cases, diarrhea. Uncomplicated recoveries usually occurred within a week following onset. The etiologic relationship of BEV to these clinical manifestations has not been determined. However, a significant rise in neutralizing antibody titers against BEV has been established subsequent to recovery in several of the affected animals.[2]

The neutralization of BEV by serum from cattle convalescing from infectious bovine rhinotracheitis (IBR) has been reported,[2] indicating serologic relationships between the two viruses. This is a report of a further study of the serologic relationship between BEV and IBR viruses based on cell culture cross-neutralization tests using immune rabbit serums.

The IBR virus used in tests conducted in this laboratory was isolated from the trachea of a calf that had died from IBR in a feed lot near Yakima, Washington. The virus culture was identified in neutralization tests using known IBR immune serums. The sources of the two BEV (BE-1 and BE-180) used have been described previously.[3]

Immune rabbit serums were produced against each bovine enterovirus. Three intravenous in-

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TABLE 1—Results of Serum Dilution-Constant Virus Method

Viruses (constant)	Serums (varying)				
	BE-1	BE-180	IBRW4	IBR297	Normal
BE-1	2.2 *	1.9	1.3	1.2	< 0.6
BE-180	1.2	2.2	2.5	2.2	< 0.6
IBR	< 0.6	< 0.6	1.8	1.7	< 0.6

* Underlined numbers represent homologous titers.

TABLE 2—Results of Virus Dilution-Constant Virus Method

Viruses (varying)	Serums (constant)			
	BE-1	BE-180	IBRW4	IBR297
BE-1	6.2 *	6.2	2.5	0.4
BE-180	6.3	6.3	2.8	3.2
IBR	0.0	0.0	2.1	2.1

* Underlined numbers represent homologous titers.

jections (2.0 ml. each of infected cell culture fluid containing 10^6 to $10^{7.5}$ t.c.d.$_{50}$ per milliliter) were given to each rabbit at ten- to 14-day intervals. The serums were collected 14 days after the third injection. The IBR rabbit immune serums were supplied by Dr. T. L. Chow, College of Veterinary Medicine, Colorado State University, Fort Collins.

Neutralizing antibody titers were determined in cell-culture neutralization tests according to the method described by Paul and Melnick for paired poliomyelitis serums,[3] using fivefold dilutions of serums and constant amounts of virus or tenfold dilutions of virus and constant amounts of serum. The log t.c.d.$_{50}$ was calculated by the method of Reed and Muench.[4] The results of the serum dilution-constant virus method are presented (table 1).

The two IBR immune serums inhibited both BEV cultures. Both IBR immune serums inhibited BE-180 to the same degree as its homologous serum and BE-1 to a somewhat lower degree. There was no significant inhibition of IBR virus by the two BEV immune rabbit serums used. The results of the virus dilution-constant serum method are presented (table 2).

IBR immune serum W-4 inhibited both BE-1 and BE-180, and IBR immune serum F-297 inhibited only BE-180. The log t.c.d.$_{50}$ neutralizing titers of both IBR immune serums were significantly lower than the homologous and heterologous BEV immune serum titers. There was no inhibition of the IBR virus by the two BEV immune serums.

The data presented indicate a serologic relationship between BEV and IBR virus. The inhibition of BEV by IBR immune serums and the lack of IBR virus inhibition by BEV immune serums indicate that the IBR virus contained one or more cross-reacting actigenic components not present in the BEV tested. These results also imply that immunization of animals against IBR would also afford protection against possible ill effects from some BEV infections.

References

[1] Moll, T., and Finlayson, A. V.: Isolation of Cytopathogenic Viral Agent from Feces of Cattle. Science, 126, (1957): 401–402.

[2] Moll, T., and Davis, A. D.: Isolation and Characterization of Cytopathogenic Enteroviruses from Cattle with Respiratory Disease. Am. J. Vet. Res., 20, (Jan., 1959): 27–32.

[3] Paul, J. R., and Melnick, J. L.: Diagnostic Procedures for Virus and Richettsial Diseases. 2nd ed., Am. Public Health Assoc. Press, New York, N. Y., (1956): 53.

[4] Reed, L. J., and Muench, H. A.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

SUMMARIO IN INTERLINGUA

Relation Serologic Inter Enterovirus Bovin e Virus de Infectiose Rhinotracheitis Bovin

Esseva previemente reportate le neutralisation de varie racias de enterovirus bovin per sero ab bestial in reconvalescentia ab infectiose rhinotracheitis bovin. Isto indica un relation serologic inter le virus de infectiose rhinotracheitis bovin e enterovirus bovin. Le presente studio es un investigation additional de ille relation per medio de tests de neutralisation cruciate de cytoculturas con le uso de immun sero de conilio.

Le methodo usate es describite in detalio.

Esseva constatate un inhibition de enterovirus bovin per sero immun a virus de infectiose rhinotracheitis bovin sed nulle inhibition de virus de infectiose rhinotracheitis bovin per enterovirus bovin.

Pathogenicitate de enterovirus bovin ha non essite demonstrate definitivemente. Sed, secundo un previemente publicate studio, le greges ab que le isolatos de enterovirus bovin esseva obtenite se distingueva invariabilemente per un anamnese de leve acute morbo respiratori in vitellos. Le presente studio suggere le possibilitate que un immunisation contra infectiose rhinotracheitis bovin constitue simultaneemente un immunisation contra le supra-mentionate morbo respiratori.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

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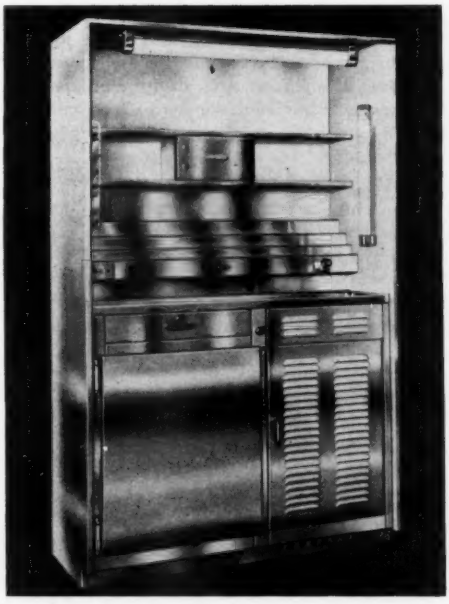
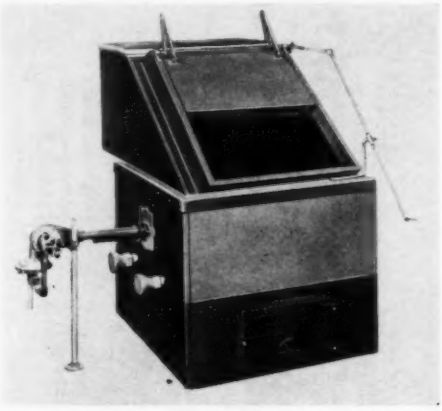


Incinerators with No Refractories

Incinerators, complete except for the refractory lining, are now available from Winnen Incinerator Co. Since the heavy refractory lining (cast in 4 complete sections) plus the base sections account for 50 to 80 per cent of the total weight, the buyer can save considerably on shipping costs by having a local mason build the lining with standard firebrick and heat-resistant mortar. Complete, well-illustrated directions are sent for the brick mason to follow.

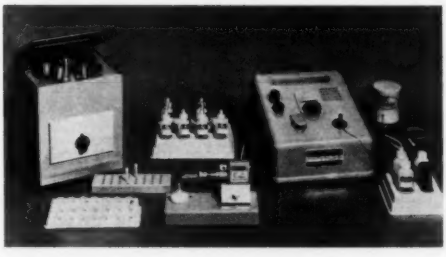


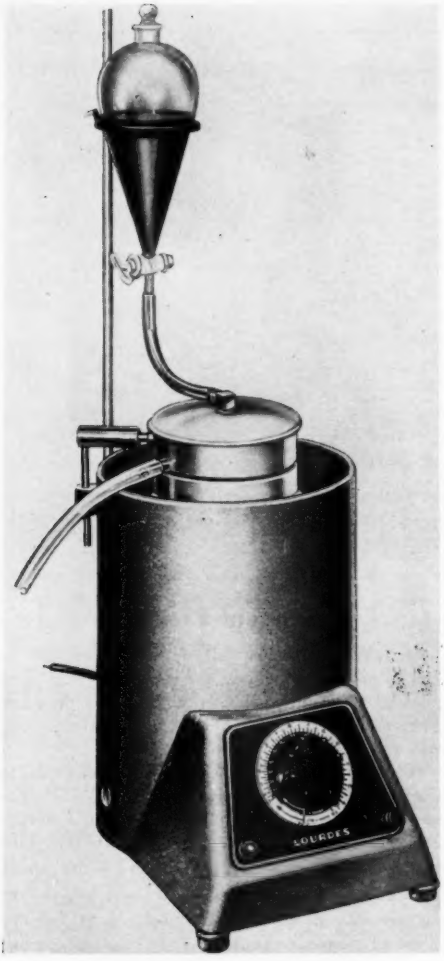
Analytical Program

Analysis of samples, 20 microliters or less in size, is now possible with the new analytical program by Coleman Instruments, Inc. The program performs 12 accurate and reliable clinical analyses with samples in the ultramicro range.

Designed to integrate with the Coleman Junior spectrophotometer, the program includes all instruments, equipment, reagents, and glassware necessary for ultramicro analysis. Key to the program is a special self-emptying ultramicro cuvette which requires only 100 microliters of sample solution but provides a full 1.00-cm. light path for the spectrophotometer beam. The cuvette carrier orients the optical faces of the cuvette with respect to the spectrophotometer. The carrier slips out of the spectrophotometer well, adapting the instrument to analysis with samples of other sizes.

Other elements in the program are a specialized microcentrifuge, a precision ultramicro titrator, and sets of reagents and volumetric dispensing equipment for 12 analytical procedures.





Bench-Top Centrifuge

A bench-model centrifuge, manufactured by Lourdes Instrument Corp., features a built-in safety shield and can accommodate five interchangeable rotors for batch or continuous flow separations.

All five heads may be spun at low or high speed to a maximum of 17,000 r.p.m. and forces as great as 35,000 x g. Two of the rotors are of conventional angle type (34°) with capacities of 400 ml. (8 x 50 ml.) and 360 ml. (24 x 15 ml.). Both permit the use of adapters to accommodate tubes as small as 1 ml. Another is a new zonal separation rotor with an 80-degree angle and with a capacity of 112 ml. (16 x 7 ml.).

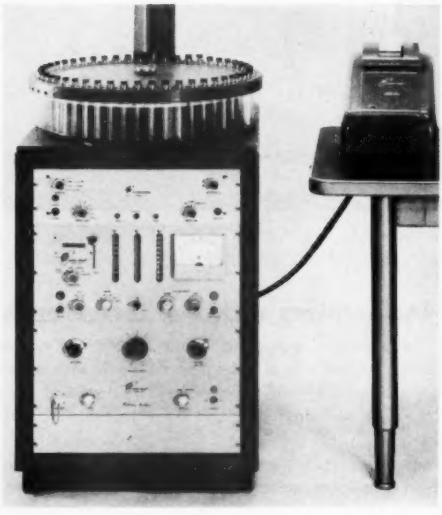
The other two rotors are for continuous flow separation. All are fitted with removable polyethylene liners which can be replaced in seconds to permit continued operation with little delay. Used with continuous flow attachments, these rotors provide for the continuous separation and concentration of sediment from mixtures with a large volume of fluid. The smaller of the two rotors has a bowl capacity of 300 ml. and can collect from 2 to 100 cc. of sediment, while the larger has a bowl capacity of 800 ml. and can collect up to 650 cc. of sediment.

This centrifuge employs a ½-hp. series-wound, ball-bearing motor with automatic self-centering. Speed is controlled by a variable transformer enclosed in a cast iron base together with a built-in line switch, a pilot light, and fuse. A heavy steel guard bowl protects the revolving head. All bearings in the centrifuge are permanently lubricated.

Automatic Sample Changer

A new automatic sample changer for use with well-type scintillation detectors can hold 49 test tubes containing gamma-emitting liquid or solid samples. The instrument, produced by Nuclear-Chicago Corporation, automatically lowers the test tubes in proper sequence into the well crystal of a shielded scintillation detector for measurement. The changer is used with standard scalers and well detectors which are available in most radioisotope laboratories.

At the end of each sample measurement, a printing timer records the sample identity number and the length of time required to reach a preset count. The procedure is repeated until all samples have been counted automatically. The system can be left unattended during the day, overnight, or on weekends, and the printed data can be collected when convenient.



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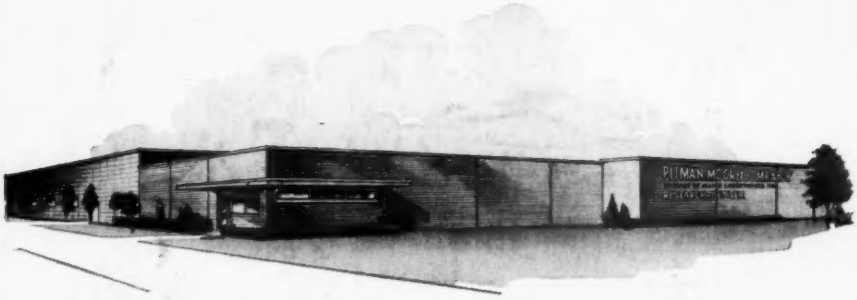

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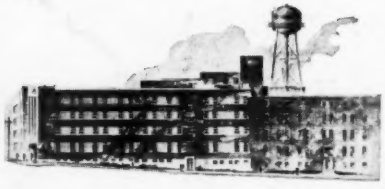

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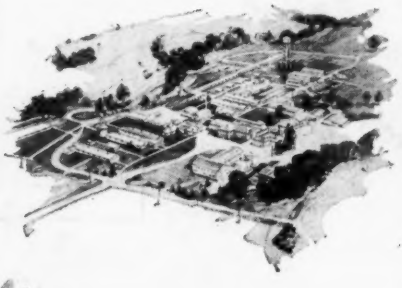

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